

# **STUDIES OF CELL-ON-CHIP TECHNOLOGY AND BASOPHIL REGULATION FOR IMPROVED ALLERGY DIAGNOSTICS**

FRIDA KALM

Doctoral Thesis

Karolinska Institutet  
Department of Clinical Science and Education  
Södersjukhuset  
SE-118 83 Stockholm

KTH Royal Institute of Technology  
School of Engineering Sciences in Chemistry, Biotechnology and Health  
Department of Protein Sciences  
Division of Nanobiotechnology  
Science for Life Laboratory, Solna  
SE-171 65 Stockholm

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# **Studies of cell-on-chip technology and basophil regulation for improved allergy diagnostics**

## **THESIS FOR DOCTORAL DEGREE (Ph.D.)**

By

**Frida Kalm**

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*Principal Supervisor:*

Associate professor Anna Nopp Scherman  
Karolinska Institutet  
Department of Clinical Science and Education

*Co-supervisor(s):*

Professor Aman Russom  
Royal Institute of Technology  
Department of Protein Sciences  
Division of Nanobiotechnology

Professor Joachim Lundahl  
Karolinska Institutet  
Department of Clinical Science and Education

Associate professor Caroline Nilsson  
Karolinska Institutet  
Department of Clinical Science and Education

*Opponent:*

Associate professor Stephanie Descroix  
Institut Curie

*Examination Board:*

Associate professor Guro Gafvelin  
Karolinska Institutet  
Department of Clinical Neuroscience

Professor Maria Tenje  
Uppsala University  
Department of Materials Science and Engineering  
Division of Microsystems Technology

Professor Lennart Nilsson  
Linköping University  
Department of Clinical and Experimental  
Medicine



*To my family*



## ABSTRACT

Allergic diseases affect approximately 30% of adults and has an impact on both the individual's quality of life as well as an economic impact on society. Two effector cells involved in allergic disease are mast cells and basophils, where basophils are more readily available in blood and therefore of great interest when studying allergy. Basophils can be recruited into the tissue during inflammation originating from for example allergic reactions or parasite infections. Allergy diagnostics starts with evaluation of the patient's medical history followed by in vivo and/or in vitro testing. All diagnostic tests have different advantages and disadvantages are chosen depending on the patient and the circumstances. In vivo tests include the gold standard of allergy diagnostics, which is the challenge tests, but also the commonly used skin prick test (SPT). Allergy diagnostics can also be done in vitro using allergen-specific IgE antibody assays and the basophil activation test (BAT). BAT is useful to study the cellular response to an allergen but is only available at university hospitals and therefore often require long transportations of blood samples. A research field that is growing fast is microfluidics which can miniaturize and improve existing methods and diagnostic tests. The aims of this thesis were to improve the existing BAT using microfluidic techniques to enable fast and cheap point-of-care (POC) diagnostics as well as to further study basophil adhesion and activation for a better understanding of basophil behavior and regulation both in vivo and in vitro in a microfluidic chip. In **paper I**, we developed a novel microfluidic immunoaffinity-based basophil activation test (miBAT) assay to investigate whether it was possible to capture and activate basophils from whole blood in a microfluidic chip. The yield of captured basophils from whole blood was 64% at a capture flow rate of 3  $\mu\text{l}/\text{min}$ . The captured basophils were activated using an anti-Fc $\epsilon$ RI antibody and the basophil identification marker CD203c and the activation marker CD63 were detected using fluorescence microscopy. This was done using blood from both healthy donors and allergic patients and showed comparable results between BAT and miBAT. In **paper II**, we further investigated whether it was possible to detect a dose-dependent allergen activation for basophils captured in a microfluidic chip. We detected a significant difference in CD63-expression between the negative control and allergen-activated basophils from allergic patients but no difference between the negative control and the non-relevant allergen (an allergen to which the patient had no IgE antibodies). The healthy donors showed no significant difference in activation between the negative control and the allergens. The miBAT results were comparable to BAT. In **paper III**, we studied basophil adhesion and activation to better understand both basophil function as well as the effect that basophil capture and stimulation in a microfluidic chip has on the cell. The basophil capture in a microfluidic chip could potentially mimic basophil adhesion to the endothelium and was therefore of interest due to the elevated background activation seen in unstimulated basophils captured in a microfluidic chip, reported in paper I and II. Basophils did not upregulate CD63 after passage through a microfluidic chip, but there was a slight but significant activation after crosslinking of CD203c, which is the surface marker used for basophil-specific capture in miBAT, giving one potential factor for the background activation. IgE-dependent (anti-IgE) basophil degranulation after crosslinking of adhesion molecules, to mimic adhesion before transmigration into tissue, showed a significant decrease in CD63-expression compared to anti-IgE activation, which indicate a regulatory function. Cytokine stimulation followed by IgE-independent (fMLP) basophil degranulation on the other hand showed a significantly increased CD63-expression compared to non-primed fMLP activation.

In conclusion, we have developed a novel microfluidic-based technique (miBAT) able to detect basophil activation (CD63-expression) using allergens in allergic patients. miBAT was also able to discriminate between relevant and non-relevant allergen activation as well as between allergic patients and healthy controls. miBAT has the potential to be used at POC for allergy diagnostics. We have also shown that crosslinking of CD203c is a potential contributor to the basophil activation seen in the negative controls in miBAT but also that IgE-dependent activation is downregulated from crosslinking of some adhesion molecules, which is of interest both in the microfluidic chip but also in vivo to better understand basophil functions.



## LIST OF SCIENTIFIC PAPERS

- I. Z. Aljadi, **F. Kalm**, H. Ramachandraiah, A. Nopp, J. Lundahl, and A. Russom  
Microfluidic immunoaffinity basophil activation test for point-of-care allergy diagnosis  
The Journal of Applied Laboratory Medicine, 2019, 4(2): p. 152.  
Published manuscript
- II. Z. Aljadi\*, **F. Kalm\***, C. Nilsson, O. Winqvist, A. Russom, J. Lundahl, and A. Nopp  
A novel tool for clinical diagnosis of allergy operating a Microfluidic Immunoaffinity Basophil Activation Test Technique  
\*Shared first author  
Clinical Immunology, 2019, 209: 108268.  
Published manuscript
- III. **F. Kalm**, L. Mansouri, A. Russom, J. Lundahl and A. Nopp  
Adhesion molecule crosslinking and cytokine exposure modulate IgE and non-IgE- dependent basophil activation  
Submitted manuscript

## SCIENTIFIC PAPER NOT INCLUDED IN THE THESIS

- I. J. Brandstrom, M. Vetander, G. Lilja, SG. Johansson, AC. Sundqvist, **F. Kalm**, C. Nilsson, A. Nopp  
Individually dosed omalizumab: an effective treatment for severe peanut allergy.  
Clinical and Experimental Allergy, 2017, 47(4): p. 40-550.  
Published manuscript



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## LIST OF ABBREVIATIONS

AIT	Allergen immunotherapy
AND	Anaphylactic degranulation
APC	Antigen presenting cell
BAT	Basophil activation test
BSA	Bovine serum albumin
C3a	Complement component 3a
C5a	Complement component 5a
CAD	Computer-aided design
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CD-sens	Basophil allergen threshold sensitivity
CR3	Complement receptor 3
CRD	Component resolved diagnostics
CTC	Circulating tumor cell
DC	Dendritic cell
DLD	Deterministic lateral displacement
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EpCAM	Epithelial cell adhesion molecule
Fc	Fragment crystallizable
FcεR	Fc epsilon receptor
FcRγ	Fc receptor common γ-chain
fMLP	N-formylmethionyl-leucyl-phenylalanine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMBS	4-Maleimidobutyric acid N-hydroxysuccinimide ester

HIV	Human immunodeficiency virus
Ig	Immunoglobulin
IgE-ab	IgE-antibody
IL	Interleukin
LOAD	Lab-on-a-disc
MFI	Mean fluorescence intensity
miBAT	Microfluidic immunoaffinity basophil activation test
NK cell	Natural killer cell
PDMS	Polydimethylsiloxane
PMD	Piecemeal degranulation
POC	Point-of-care
RANTES	Regulated on activation normal T cell expressed and secreted
Re	Reynold's number
RPMI	Roswell Park Memorial Institute
SPR	Surface plasmon resonance
SPT	Skin prick test
Th2 cell	T helper 2 cell
TLR	Toll like receptor
VLA-4	Very late antigen 4
%CD63+	Percent positive CD63

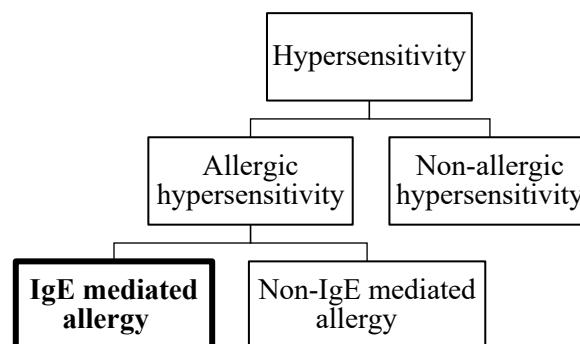
# 1 INTRODUCTION

## 1.1 Allergy

Allergic diseases affect approximately 30% of adults and 40% of children globally, which means that the effect of the disease has a noticeable impact on most people's lives as well as on the society [1]. In a study on allergic rhinitis it was estimated that the cost was €1.4 billion annually in Sweden [2], and in a Swedish study on allergies towards staple foods the cost was estimated at €8164 per year higher in households with one or more food allergic adult [3]. Neither of these studies takes into account the additional cost of additional forms of allergic diseases nor the psychosocial effects involved such as problems with sleep, fatigue during pollen season and anxiety for severe food allergic reactions [4]. Both allergic adults and children show a loss of productivity at work and in school, especially during pollen season [5, 6]. In a study in the United States the loss of worker productivity due to their health conditions was compared and those with allergic rhinitis had a lower productivity than workers with e.g. high stress, high blood pressure, diabetes or coronary disease [7].

Allergy is defined as “a hypersensitivity reaction initiated by immunological mechanisms” [8] can be divided into allergic hypersensitivity and non-allergic hypersensitivity. Allergic hypersensitivity can be further be divided into IgE mediated allergy and non-IgE mediated allergy [8], see Figure 1. The focus of this thesis will be on IgE mediated allergy.

Allergy occurs when the immune system responds to innocuous substances, so called allergens. An allergen is a harmless antigen that causes an abnormal immune response in the form of an allergic reaction. Allergens are often proteins or peptides associated with certain properties, which increases their allergenicity. Allergens are for example usually resistant to proteolysis, which makes them less likely to be degraded before they have a chance to be detected by the immune system. Allergens are also often glycosylated during their post-translational modification, which are thought to increase the chance of detection in the body [9]. In addition, other factors that are associated with allergens are enzymatic activity, heat stability and stability at varying pH, the latter explaining why some food allergens still cause an allergic reaction after ingestion [10].



**Figure 1.** Schematic of how hypersensitivity is divided into allergic and non-allergic hypersensitivity and how allergic hypersensitivity can be further divided into IgE mediated and non-IgE mediated allergy.

### **1.1.1 Atopy**

Atopy is the hereditary or constitutional tendency to produce IgE antibodies (IgE-abs) in response to allergens. Atopy is often inherited and a child has a 50-75% risk of developing IgE-mediated allergy if both parents are atopic compared to 10-15% if neither parent is atopic [8]. The increase in allergic diseases are thought to be due to a combination of several different factors such as environmental factors, socioeconomic status and diet [11]. The hygiene hypothesis suggests that exposure to pathogens early in life reduces the risk of allergy, which would explain why children living on e.g. farms generally are less allergic. Other factors like a western diet, pollution and use of antibiotics are thought to increase the risk of developing allergic diseases [12].

### **1.1.2 Allergic diseases**

Some of the more common forms of allergic diseases are rhino conjunctivitis, allergic asthma, atopic dermatitis and food allergy. Symptoms vary from mild symptoms such as itching in the mouth and sneezing from rhino conjunctivitis [13] to more severe reactions such as swelling of the airways and anaphylaxis from food allergy. Anaphylaxis can be fatal and food allergy is the most common cause in children and adolescents. In adults however, medicines and drugs are the leading cause of deaths from anaphylaxis. Food allergy does not always cause severe allergic reactions, but can instead cause e.g. urticaria, sneezing and vomiting [14]. In allergic asthma there is a chronic inflammation in the airways which can cause constriction leading to e.g. wheezing and shortness of breath [15]. Atopic dermatitis is a chronic inflammation in the skin characterized by red, dry and itching skin [16].

## **1.2 Immunology of IgE-mediated allergy**

IgE mediated allergy can be divided into a sensitization phase and an effector phase where the effector phase can be further divided into the immediate and the late response. Allergic disease only occurs when sensitization leads to allergic symptoms, which is not always the case.

### **1.2.1 The sensitization phase**

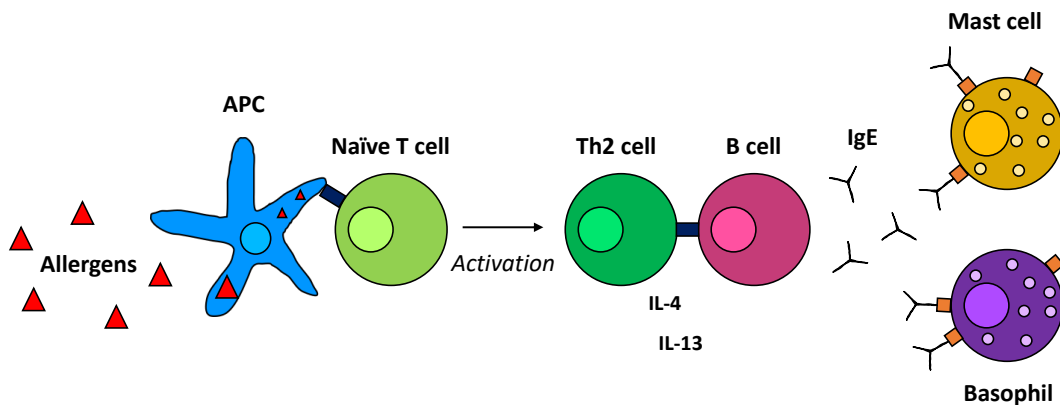
At the first encounter with an allergen a sensitization against the allergen can occur; the allergen is then processed and presented by major histocompatibility complex II on an antigen presenting cell (APC), e.g. a dendritic cell (DC), to a naïve T cell. The naïve T cell then becomes a T helper 2 cell (Th2 cell), in presence of the cytokine interleukin 4 (IL-4) and produces cytokines such as IL-4 and IL-13. In addition, in presence of IL-4 the Th2 cell interacts with cluster of differentiation (CD) 40 on the B cell via the CD40-ligand on the Th2 cell and switches the B cell into an IgE producing cell, specific to the allergen [17]. These polyclonal allergen-specific IgE-abs will then either bind to the high affinity IgE-receptor (FcεRI) on the surface of mast cells and basophils or circulate in free form [11, 17, 18], see Figure 2.

### **1.2.2 IgE**

IgE was first discovered in 1967 by two from each other independent research groups, one in Uppsala, Sweden and one in Denver, Colorado, USA. IgE is present at the lowest concentration out of the immunoglobulin subtypes at approximately  $5 \times 10^{-5}$  mg/ml. IgE also has the shortest half-life of the immunoglobulins at two-three days [19]. The expression of IgE is generally higher in allergic patients and during parasite infection. There are two receptors that can bind



IgE, the low affinity receptor FcεRII present on B-cells and the high affinity receptor FcεRI found on mast cells and basophils [20].



**Figure 2.** Sensitization to an allergen. The allergen is taken up and processed by an antigen presenting cell (APC) before being presented to a naïve CD4<sup>+</sup> T cell, which is then activated into a T helper 2 (Th2) cell. The Th2 cell secretes IL-4 and IL-13 and switches the B cell into an IgE producing B cell. The produced IgE can then bind to the IgE-receptor on mast cells and basophils.

### 1.2.3 The high affinity IgE-receptor, FcεRI

FcεRI is a tetramer on the surface of basophils and mast cells consisting of an alpha, a beta and two gamma chains. The gamma and beta chains are for maturation and transport of the receptor, whereas the alpha chain contains two extracellular asymmetric binding sites and binds the fragment crystallizable (Fc) region of the IgE-ab. The beta and gamma regions are phosphorylated upon crosslinking of IgE and signals for activation and degranulation of the cell [21]. There is an equilibrium between free IgE-abs and FcεRI expression on mast cells and basophils since IgE stabilizes FcεRI, which means that a reduction in free IgE leads to a reduction in FcεRI expression [21].

### 1.2.4 The effector phase

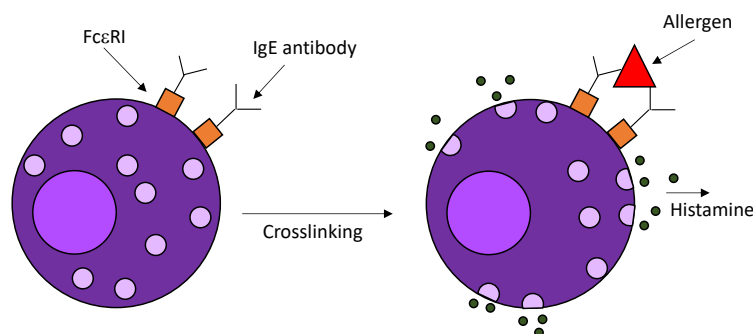
In the effector phase, when re-exposed to the same allergen, the allergen will bind to and crosslink IgE-abs on mast cells or basophils, which in turn will cause degranulation of the cell releasing mediators such as histamine, proteoglycans, IL-4 and IL-13 [11, 17]. Histamine is an influential mediator of allergic reactions since it binds the histamine receptors (H1 and H2), which mediates smooth muscle contractions and increases the permeability of blood vessels causing for example swelling, itching or hypotension, the latter which can lead to anaphylaxis [17, 22].

There can also be a delayed allergic response initiated several hours after allergen exposure where the cells such as Th2 cells, eosinophils and basophils have been recruited and subsequently activated [11, 23].

### 1.2.5 The mast cell

Mast cells are an important effector cell in allergic reactions and are mainly found in tissue around blood vessels and epithelial cell surfaces. Mast cells are thought to play a role in skin protection from bacteria and parasites, but also in wound healing. Mast cells are associated with diseases such as mastocytosis [24] and allergy. There are different subtypes, i.e. connective tissue mast cells and mucosal mast cells, but they can all be activated through allergen crosslinking via IgE-abs on the surface of the cell causing degranulation, see Figure 3.

Mast cells have readily made mediators that are released immediately upon IgE-activation, such as histamine, tryptase and proteoglycans [17, 25]. Mast cells are the main initiator of allergic symptoms, due to their presence and early allergen exposure in the gut, airways and skin. Mediators released from mast cells can recruit other cells to the tissue in response to inflammation and have been reported to produce IL-33 upon activation, which in turn recruits basophils during late-phase allergic inflammation [26].



**Figure 3.** Mast cell or basophil degranulation and release of histamine after allergen crosslinking of IgE-abs bound to the high affinity IgE-receptor (FcεRI) on the cell surface.

### 1.2.6 The basophil

Basophils are rare granulocytes, which represent less than one percent of peripheral blood leukocytes and originate from CD34+ myeloid hematopoietic progenitors in the bone marrow. Basophils are short-lived and circulate approximately 60 hours in blood. Basophils have been found to have a regulatory role in both innate and acquired immunity and can be recruited into the tissue during inflammation. Basophils are involved in a variety of functions including the protective immunity against helminths and Th2 differentiation, but are also implicated in diseases such as allergy, autoimmunity and cancer [27, 28].

#### 1.2.6.1 Basophil functions

Basophils are major IL-4 producing cells in response to activation and influence the Th2 response by differentiate naïve T cells into Th2 cells [29]. IL-13 secreted from basophils play a role in B-cell class switching to IgE synthesis in allergen sensitization [30]. Basophils have been found to function as potential APCs during priming of Th2 response in mice [31, 32]. For human basophils on the other hand, this does not seem to be the case [33-35].

#### 1.2.6.2 Growth factors for basophils

The most potent growth factor for basophils is IL-3, which has a critical role during basophil development, can block apoptosis and prime the cell. Basophils have an autocrine mechanism for IL-3, since they can both produce and secrete IL-3, upon IgE-dependent activation, while also expressing the IL-3 receptor (CD123) [36]. IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are similar to IL-3 as growth factors but have a weaker effect on basophils than IL-3 [37].

### 1.2.7 Basophil degranulation

Degranulation of basophils can occur via two pathways, piecemeal degranulation (PMD) or anaphylactic degranulation (AND). In PMD, small vesicles are formed containing small amounts of e.g. pre-formed histamine, which are released upon degranulation. PMD is thought to play a role in paracrine and endocrine cell signaling [38]. In AND there are granules releasing

larger amounts of pre-formed histamine, leukotrienes, prostaglandins, IL-4 and IL-13. For mast cells, AND is considered an immediate cellular response to parasite infection [39] and it is therefore likely that basophil AND has a similar function. Histamine released from basophils widens blood vessels near the site of inflammation, allowing better access for cells to enter into the tissue. Histamine is a strong chemoattractant and is involved in the recruitment of for example mast cells and eosinophils [40].

#### ***1.2.7.1 Degranulation surface markers***

CD63 is a membrane protein that belongs to the tetraspanin family and has been seen to be involved in the regulation of cell development, growth, activation, and motility by interacting with integrins. CD63 is highly expressed on the granule membrane in basophils, but at a very low level or not at all in the small vesicles. Both the small vesicles and the granules fuse with the plasma membrane, which if present, will expose CD63 on the basophil cell surface. CD63 is the most commonly used surface marker for basophil activation and degranulation since it is only highly expressed after AND [41].

CD203c is an ecto-nucleotide pyrophosphatase/phosphodiesterase with unknown purpose. CD203c is only expressed on basophils and mast cells and is therefore suitable as an identification marker for basophils in blood. Since CD203c is also rapidly upregulated on basophils after both piecemeal and anaphylactic degranulation it is also commonly used as an activation marker [41-43].

### **1.2.8 Basophil activation**

Basophil activation can occur via crosslinking of antibodies on the surface of the cell, from products from the complement system, via cytokine stimulation or from bacteria-derived products. Basophil activation can cause degranulation but also production of cytokines such as IL-3, IL-4, IL-6, IL-8, IL-13 and RANTES.

#### ***1.2.8.1 Antibody-dependent basophil activation***

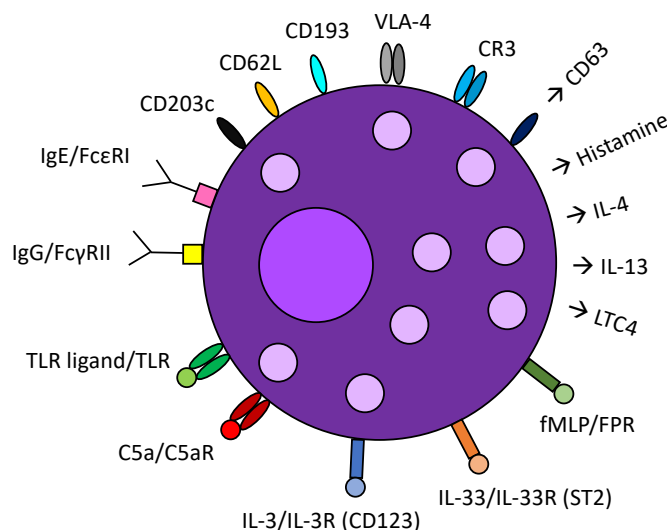
Basophils are activated via allergen crosslinking to IgE-antibodies bound to FcεRI on the surface of the cell, see Figure 3. The activated basophil immediately releases histamine during AND, as well as other preformed mediators such as proteoglycans and IL-4 and IL-13 [30]. Basophils also express FcγRIIA (CD32a) and FcγRIIB (CD32b), which are receptors for IgG. CD32a is an activating receptor subtype and CD32b an inhibitory receptor subtype. Binding to CD32a induces a slight activation with CD203c upregulation in basophils after IL-3 priming while binding to CD32b prevents IgE-dependent activation [44].

#### ***1.2.8.2 Antibody-independent basophil activation***

Basophils can also be activated via cytokines such as IL-3, IL-33, IL-8, IL-5 and GM-CSF, by bacteria derived products such as fMLP, by toll-like receptor (TLR) ligands [45] or via the complement system [46], see Figure 4. Antibody-independent basophil activation does generally not result in AND.

Basophils secrete IL-13 in response to IL-3 stimulation and IL-4 secretion is elevated from IL-3 priming before IgE-dependent activation [36]. IL-33 is an alarmin that is released by e.g. endothelial cells upon cell damage [47] and is elevated in asthma patients [48]. IL-33 induces IL-4, IL-8 and IL-13 secretion from basophils in presence of IL-3 or after IgE-dependent activation [49, 50]. fMLP is a strong leukocyte chemoattractant derived from bacteria and

causes AND in human basophils [51]. TLR activation causes the basophil to produce cytokines such as IL-4, IL-13, IL-8 or RANTES, depending on which of the different TLRs that is triggered [52]. In addition, complement component 3a (C3a) and C5a are complementary factors that can cause AND in basophils after IL-3 priming and are part of the late phase allergic response [46]. More studies are needed to better understand human basophil function and activation.



**Figure 4.** Basophil expression of surface markers and receptors involved in activation and recruitment to tissue as well as release of mediators from basophil activation.

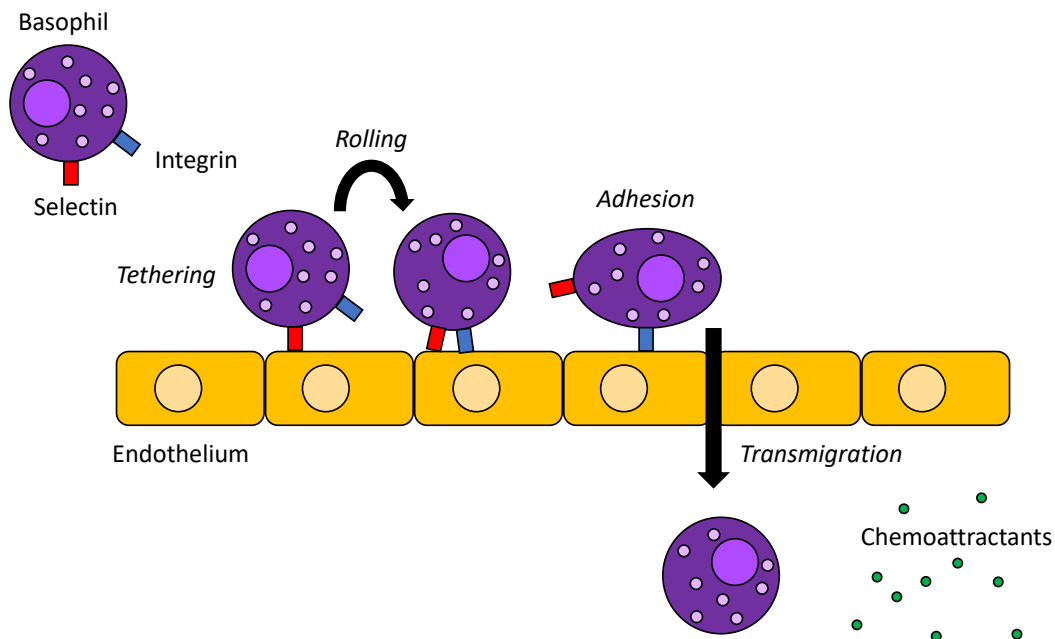
### 1.3 Basophil adhesion and transmigration

Basophils in circulation are recruited into tissue during inflammation caused by e.g. parasite infection or in response to allergic reactions [53]. First, cells at the site of inflammation will release chemokines/cytokines that causes the endothelium to express adhesion molecules to attract the basophils in blood. The basophil express adhesion molecules that are involved in the different steps during; rolling adhesion, tight adhesion and endothelial transmigration and chemoattraction [54]. The basophils will bind to ligands on the endothelium via selectins causing a rolling motion while the cytokines expressed also upregulates the integrins on the cell, causing tight adhesion. The basophil then migrates through the endothelium in a process called transmigration. Once in the tissue, the basophil move along the chemotactic gradient towards the site of inflammation where they can signal for recruitment of more inflammatory cells or degranulate to try to eliminate the cause of the inflammation [54] (Figure 5).

#### 1.3.1 Cytokines and chemoattractants

Eotaxin (C-C motif chemokine ligand 11 (CCL11)) and RANTES belong to the most potent chemoattractants for basophils and binds the eotaxin receptor CD193 [55]. Other cytokines that induce basophil transmigration are for example IL-3, IL-4, IL-5, IL-33, IL-8 and GM-CSF [56]. IL-33 is a pro-inflammatory cytokine secreted by the endothelium upon inflammation and will upregulate CD11b expression on basophils, which suggests that IL-33 can regulate basophil binding to the endothelium and play a role in transmigration [37]. Cells such as macrophages, DCs, eosinophils, mast cells and B-cells can produce IL-33 upon allergen driven-inflammation to recruit for example basophils. Basophils can secrete cytokines such as IL-8 in

presence of IL-3 upon recruitment, which in turn can recruit neutrophils for late phase inflammation in IgE-mediated allergy [57]. IL-3 is a major growth factor for basophils, but also involved in recruitment via CD193 upregulation [56, 58]. CD4<sup>+</sup> T cells and regulatory T cells can secrete IL-3 and thus recruit basophils during parasite infection [59].



**Figure 5.** Basophil tethering, rolling, adhesion and transmigration from the blood vessel and through the endothelium in response to chemoattractants released due to inflammation in tissue.

### 1.3.2. Adhesion molecules

L-selectin (CD62L) and very late antigen 4 (VLA-4, CD49d/CD29) are involved during basophil tethering and rolling on the endothelium. CD62L is a selectin that is not downregulated on basophils in the same way as it is on neutrophils upon activation [60]. The CD62L shedding on leukocytes is regulated by the cytoplasmic tail but also by mechanical shedding during rolling [61]. VLA-4 is a  $\beta 1$  integrin and is involved in both tethering, rolling and adhesion. VLA-4 binds fibronectin or vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells during chemotaxis [62]. In basophil adhesion, the complement receptor 3 (CR3, CD11b/CD18) is a  $\beta 2$  integrin involved in tight adhesion and binds to ligands such as fibronectin or intercellular adhesion molecule-1 (ICAM-1) on endothelial cells [53, 63].

## 1.4 Allergy treatment

There are many symptomatic treatments available for allergy, such as antihistamines and corticosteroids. Antihistamines block released histamine from binding to its receptor and thus prevent allergic symptoms [64] and corticosteroids binds their corticosteroid receptors, which blocks the transcription of e.g. pro-inflammatory cytokines involved in the immune response to allergens [65]. Another allergy therapy is an anti-IgE-ab (omalizumab) that reduces the immune response to allergens. The anti-IgE-ab binds free circulating IgE-ab and prevents them from binding to the Fc $\epsilon$ RI on the surface of mast cells and basophils. There is always a balance between the amount of free circulating IgE molecules and the amount of Fc $\epsilon$ RI expressed on the cell surface of mast cells and basophils, since IgE stabilizes the receptor [21, 66]. The

binding of free circulating IgE molecules by omalizumab will lead to a downregulation of the expression of FcεRI.

There are also therapies that aim to cure allergic diseases, such as allergen immunotherapy (AIT). In AIT the patient is exposed to the allergen, starting with a very small amount that is increased over time. AIT is the only therapy that may lead to desensitization or tolerance, at this time, and the most common AIT is towards pollen allergy. AIT and omalizumab can also be combined to reduce the risk of adverse allergic reactions during treatment and has been used for e.g. cow's milk allergy [67] and peanut allergy [68, 69].

## **1.5 Allergy diagnostics**

Allergies are very common worldwide, which means that methods for fast, cheap, and accurate diagnostics are needed. Diagnostic methods are also important to monitor allergy treatment i.e. companion diagnostics. There are different diagnostic tools available, but further improvements are required to make the healthcare system more efficient.

### **1.5.1 In vivo diagnostic allergy tests**

#### **1.5.1.1 Skin prick test**

The skin prick test (SPT) is a cheap and fast in vivo method to determine whether a patient is sensitized to one or several allergens but does not always correlate to clinical allergy. An allergen extract is usually applied to the skin on the volar side of the arm and is delivered into the skin by inserting a lancet through the allergen. If there is allergen-specific IgE-ab present on the mast cells in the skin, there will be a local allergic reaction where mast cells are activated due to allergen crosslinking and mediators such as histamine are released. Histamine causes an inflammation in the area, which leads to wheal formation. If the wheal is 3 mm in diameter or larger within 15 minutes, the result is considered positive [70]. The result of SPT can vary due to the allergen extract used and who is performing the SPT. Also, the outcome will be affected if the patient is taking antihistamines, since the histamine is prevented from bind to its receptor and from forming a wheal of the correct size. If the patient is severely allergic, SPT could potentially cause a systemic allergic reaction, but this is rare. SPT is commonly used in the clinic and is useful for fast and cheap allergy diagnostics, but often require verification via an additional diagnostic test due to false positive responses [70, 71].

#### **1.5.1.2 Challenge test**

Challenges are considered the gold standard of allergy diagnostics and is most commonly used in food allergy diagnostics (oral food challenges). Challenges are most often done if patient history, analysis of SPT and allergen-specific IgE-abs have already been done but given contradicting results or to, in a safe environment, show the patient that they can or cannot ingest a certain food without adverse reactions. During a challenge, the patient is exposed to the allergen at increasing levels until objective symptoms occur or until maximal dose is reached. Oral challenges are considered accurate, when it comes to positive or negative outcome, but it is not possible to determine allergen sensitivity via a food challenge since the symptoms and allergen dose eliciting symptoms will vary at repeated challenges [72, 73]. Antihistamines and corticosteroids will affect the outcome of the challenge and must be avoided prior to the test. Due to the risk of severe allergic reactions, the test must be performed at a clinic with skilled medical personnel. Challenges are time consuming and resource demanding in terms of for example personnel and are therefore expensive. The test can be open, where both the patient

and the medical staff are aware of the allergen being tested, or blinded, where either only the medical staff or neither the medical staff nor the patient know which allergen is being tested. Open challenges are most commonly used since it is time-saving compared to blinded challenges that requires an additional challenge using placebo [73].

## **1.5.2 In vitro diagnostic allergy tests**

### **1.5.2.1 Allergen-specific IgE antibody assays**

There are several assays available that can detect sensitization (allergen-specific IgE-ab in serum) to thousands of different allergens [74]. Examples of assays are ImmunoCAP (Phadia) [75], and Immulite (Siemens) [76], where ImmunoCAP is the most commonly used IgE assay in Sweden. All assays have allergens coupled to a solid surface and if the allergen-specific IgE-abs are present in serum, they will bind to the allergen. Enzyme conjugated anti-IgE-abs will then bind to all the allergen-specific IgE-abs present, which can then be visualized after binding to a fluorescent substrate [74]. The assays can detect many allergens in parallel and do not require physical contact between the patient and the allergens. Patients who are sensitized (have allergen-specific IgE-abs) but no show no clinical symptoms, however, still show a positive result in these assays. One example of sensitized patients without symptoms are patients that have outgrown their allergy [77]. There have been some studies that connect higher levels of allergen-specific IgE-abs to clinical symptoms [78] but this is not always the case, which is why there is a need for tests that measures the effect of allergens at a for example cellular level. The sensitivity of these allergen-specific IgE-ab assays are similar to SPTs [79].

#### **1.5.2.1.1 Component resolved diagnostics**

In component resolved diagnostics (CRD), IgE-abs can be analyzed to one or more specific proteins in the allergen source and is used to improve the accuracy of allergy diagnostics, often in allergen-specific IgE-ab assays [74]. The proteins used in CRD are purified, native or recombinant. It is important to know which protein is responsible to e.g. distinguish between a systemic allergic reaction and local symptoms in the mouth caused by cross-reactivity [80].

### **1.5.2.2 Basophil activation test (BAT)**

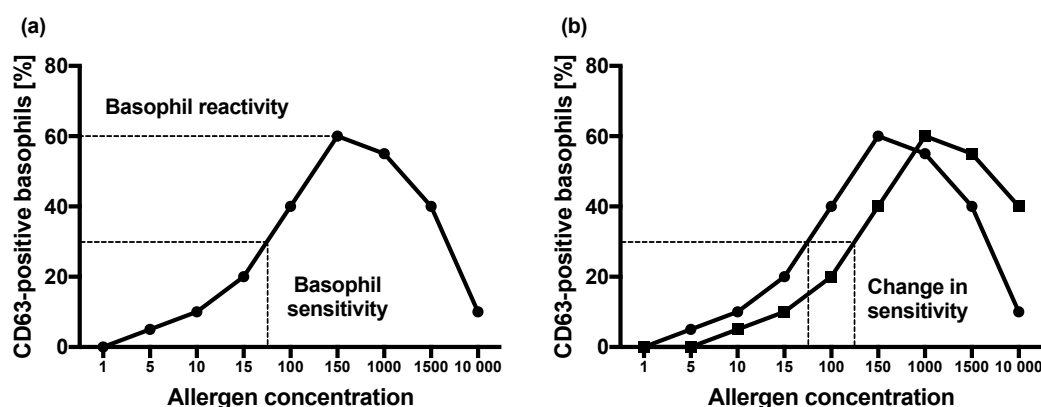
Even though mast cells are more commonly associated with allergy they cannot easily be used for in vitro diagnosis considering they are tissue bound. Basophils on the other hand are readily accessible in blood and are well suited for in vitro analysis. The basophil activation test (BAT), is a method that more closely reflects a challenge than the other in vitro diagnostic tests mentioned [81]. BAT can be thought of as a challenge in a test tube, which eradicates the risk of allergic reactions. BAT detects allergen activation of basophils in blood, either by measuring the histamine release or by detecting activation markers on the surface of the basophils. Histamine release can be measured using enzyme-linked immunosorbent assay (ELISA) or fluorimetry where the percentage of the total histamine content in the granules are detected. Basophil activation can also be analyzed using flow cytometry where activation markers are fluorescently labeled. Possible activation markers include CD203c, CD63, CD69, CD107a, CD13 and CD164 [81, 82] where CD63 and CD203c are most commonly used. CD203c is always expressed on basophils but is further upregulated after activation and degranulation. CD63 expression is closely associated with anaphylactic degranulation since the marker is present in the granules together with histamine. Several identifying markers for basophils such as CD203c, CD193, CD123, IgE and CCR2 can be used in different combinations and gating strategies [81]. CD203c is considered specific for basophils since they are the only cells in

blood that express this marker and is commonly used alone or in combination with CD193 for basophil identification [83]. CD193 can be used if the basophil population is difficult to identify using only CD203c. BAT results are not influenced by anti-histamines but systemic corticoid steroids have to be avoided since they can affect basophil activation by for example reducing histamine release [84, 85] and has also been reported to inhibit IL-4 secretion [86].

Basophils from approximately 10% of the population will not respond to IgE-ab crosslinking with AND, so called non-responders. These non-responders are not possible to evaluate using BAT [87, 88]. It is not known yet why individuals are non-responders, but some non-responders seem to have a reduced expression of the protein tyrosine kinase Syk, which is needed for IgE-mediated degranulation [89] as well as have reduced levels of free IgE and FcεRI expression on basophils [90].

#### 1.5.2.2.1 Basophil allergen threshold sensitivity, CD-sens

A clinically used version of the BAT is basophil allergen threshold sensitivity (CD-sens). CD-sens measures the allergen sensitivity towards an allergen. By activating basophils at decreasing allergen concentrations, a dose-response curve can be generated, see Figure 6. From the dose response curve, the CD-sens value is calculated from the concentration at 50% of the maximum activation, which is then inverted and multiplied with 100. This gives a value that decreases with decreased allergen sensitivity and makes it a useful tool in the clinics when monitoring patients. The information obtained from a dose-response curve will be more comprehensive than using only one or two allergen concentrations. If the allergen sensitivity decreases over time or during e.g. AIT, a higher allergen concentration will be needed for the basophil to react [88]. The concentration that gives the maximum basophil activation measures basophil reactivity, i.e. the propensity for the basophils to react and can be used to a dichotomous result (positive/negative) [88, 91, 92]. There is no relationship between the level of basophil reactivity and allergen sensitivity [91]. Basophil activation is dependent on the affinity of the allergen-specific IgE-ab to the epitope on the allergen, which means that it will vary between patients. The number of allergen-specific IgE-abs bound to the surface of the cell will affect the shape of the dose-response curve [88].



**Figure 6.** Example of a dose-response curve for CD63-positive basophils at several allergen concentrations. (a) Basophil reactivity is read as maximum response and basophil sensitivity is read as the allergen concentration at half of the maximum response. (b) A change in sensitivity is read as a shift along the x-axis.



#### **1.5.2.2.1.1 Clinical applications for CD-sens**

CD-sens has been shown to be a complement to in vivo challenges in several different types of allergies, such as allergic asthma [93], allergic rhinitis [94] and food allergies to e.g. peanut [72] and hazelnut [95]. The method can also been used to analyze the decrease in allergen sensitivity during AIT to birch, timothy [96] and hymenoptera venom [97] as well as during omalizumab treatment of allergic asthma to cat [98], peanut allergy [69] and cow's milk allergy [99].

However, there is still a need for improved diagnostic methods to meet an increasing clinical demand and one way to explore this is to use microfluidics.

### **1.6 Microfluidics**

Microfluidics is defined as “science and technologies for designing and/or fabricating devices and processes for handling and control of minute amounts of fluids and/or gases in a miniaturized system” [100] and is a rapidly growing research field.

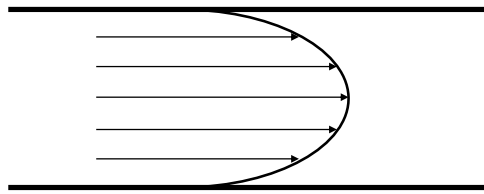
Microfluidics and lab-on-chip technologies can be used for various medical and biochemical applications [100, 101]. Microfluidic devices can be designed to make existing techniques cheaper, faster and easier to use. Due to dramatic volume reduction in microfluidics, less reagents are required which reduces the cost. Also, the sample volume will be smaller [101], which is especially beneficial when working with samples from small children or samples that are difficult to attain. Microfluidic devices are often small, which makes them portable and can be used in point-of-care (POC) settings [102, 103]. The microfluidic chips are often made from cheap materials, such as polydimethylsiloxane (PDMS) and glass [104]. Some microfluidic chips are integrated and can perform both sample processing and detection [105]. There are a lot of different applications for lab-on-chip, varying from the common pregnancy tests, which are paper-based lateral flow assays [106], to chips for rare cell-analysis [107]. In spite of all these advantages, most microfluidic devices are thus far mostly used as proof-of-concept in research and there is still a need to make the technology more user-friendly to for example clinicians [101].

#### **1.6.1 Fluid dynamics**

Fluids behave very differently at micro-scale compared to macro-scale. At macro-scale, gravitational forces dominate, whereas at micro-scale it is the capillary forces and surface tension that has the greatest effect on the fluid behavior [100, 101].

Reynolds number ( $Re$ ) is a dimensionless number that quantify the ratio between inertial forces to viscous forces and is used to predict the behavior and flow patterns of a fluid. At high  $Re$  ( $>\sim 2300$ ) inertial forces dominate and the flow is turbulent meaning that there are changes in flow velocity as well as formation of vortexes. At low  $Re$  ( $<\sim 2300$ ), viscous forces dominate leading to laminar (non-turbulent) flow, which is constant in fluid motion and where the fluid flows in streamline layers that do not disrupt each other.  $Re$  scales with the dimension and flow velocity. Hence, scaling down in dimension and/or flow velocity results in low  $Re$ . In microfluidics,  $Re$  is often smaller than 1 meaning that the flow is laminar and viscous forces dominate, which allows for precise control of a fluid in microfluidic channels [108]. At low  $Re$ , a Poiseuille flow (a pressure difference induced flow) through a straight channel will exhibit a parabolic flow profile (Figure 7), which is due to higher velocity at the center whereas the velocity at the walls approaches zero due to the no-slip condition [100, 101, 109]. The shear

stress will be the highest at the channel walls because of the friction that the wall exerts on the fluid [100].



**Figure 7.** Parabolic flow profile at low Reynold's number ( $Re$ ) in a microfluidic channel.

### 1.6.2 Microfluidics-based cell separation

Microfluidics has the potential of eliminating a number of shortcomings associated with complex sample processing at the macroscale. The ability to handle very small volumes of reagents in microchannels very precisely is promising for cell separation applications. Microfluidics can be used to manipulate fluids, particles and cells in fluids through e.g. mixing or separation [101] and can be used to isolate cells based on various cell properties using different techniques. Cell manipulation techniques can broadly be characterized as active, passive and affinity-based separation.

#### 1.6.2.1 Active separation

Active cell separation techniques require external forces such as acoustic waves, magnetic or electrical charge or optics.

In acoustofluidics, which is the combination of acoustics and microfluidics, the cells are exposed to acoustic waves that will separate cells based on size into the nodes of the acoustic waves. By adding flow, the cells can be separated based on size at different outlets [110] and has been used to for example separate prostate cancer cell line from blood [111].

In magnetophoresis the cells are separated based on their magnetic properties or by labeling the cells with magnetically labeled antibodies. Magnetophoresis has for example been used to separate hemoglobin from other blood cells and components based on their natural iron content [112, 113].

Dielectrophoresis exposes the cells to a non-uniform electric field where the cells will move depending on their polarization. The cells do not require surface charge to be able to become polarized [112]. Dielectrophoresis has been reported to be able to separate several cell types (RBCs, T-cells, platelets and human breast cancer cell line) simultaneously based on their size and polarization [114].

In optical manipulations a focused laser beam can trap a cell using the difference in refractive index between the cell and surrounding fluid, using so called optical tweezers [112]. This technique has been used in fluorescently labeled-cell sorting (FACS) on chip [115].

#### 1.6.2.2 Passive separation

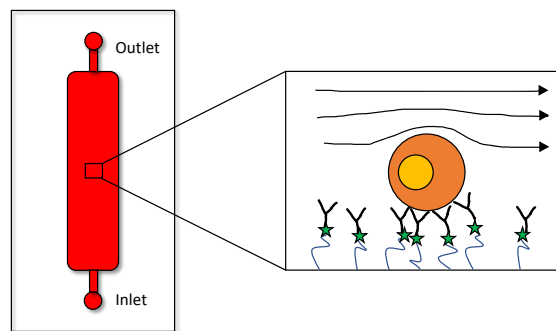
In passive separation there are no external forces, but the cells are instead separated based on their physical properties such as cell size, deformability, compressibility and shape. The separation is performed by using e.g. filters or by adjusting channel design.

Cells can be separated based on deformability and size by using a channel design that forces the cells to take different paths. One example is deterministic lateral displacement (DLD) where each row of micro-posts in an array is slightly shifted from the previous row. Smaller cells will take a more straight pathway through the chip than larger cells that will be displaced laterally, enabling separation at the outlets [116]. DLD has been used to separate e.g. cancer cells from blood [117, 118].

In inertial focusing, the cells are focused and separated at high flow rates in a straight or curved channel. The forces that separate the cells by size in curved channels are lift forces and Dean drag forces [112]. Inertial focusing has been used to separate various leukocytes [119] as well as circulating tumor cells (CTCs) [120] from blood.

### 1.6.2.3 Immunoaffinity-based isolation

Immunoaffinity-based isolation uses antibodies to specifically capture the cell of interest on the surface of the microfluidic chip via antibody binding to a cell-specific antigen (Figure 8). Antibodies can be used to capture a specific cell type from a complex solution such as whole blood, where the other cells that are not bound to the antibodies attached to the chip wall will pass through the chip. To determine the optimal shear stress a Hele-Shaw device can be used where the shear stress decreases linearly over the length of the chip, starting at maximum shear stress at the inlet [121]. The optimal shear stress allows for optimal binding between the chosen capture-antibody and the antigen on the cell and determines the capture flow rate. The shear stress also depends on the geometry of the chip design and the flow rate can be calculated from the optimal shear stress and geometry of the chip [122]. The specific capture of the cell of interest in a microfluidic chip is called positive enrichment and allows further manipulation and imaging of the chosen cell inside the chip. Negative enrichment requires no labelling or capture of the cell of interest, since the cells are collected at the outlet of the chip while the non-target cells are specifically captured using antibodies [123].



**Figure 8.** Schematic of immunoaffinity-based isolation of a cell captured in a microfluidic chip using a cell-specific capture antibody attached to the chip wall.

Immunoaffinity assays in microfluidics are commonly used to capture rare cells such as CTCs from whole blood by using immobilized anti-EpCAM antibodies [124] or to capture exosomes secreted from ovarian cancer cells [125], which possibly could be used in cancer diagnostics in the future. Using immunoaffinity instead of e.g. size-based separation for CTC-isolation means that even smaller CTCs will be captured, since CTCs are not all the same size even though they generally are larger than other cells in blood [126]. The capture and counting of CD4<sup>+</sup> T cells from whole blood for diagnosis or monitoring progression of human

immunodeficiency virus (HIV) infection has also been shown [122]. Immunoaffinity can also be used to analyze the heterogeneity of secreted proteins from captured T cells from melanoma patients [127]. A limitation of immunoaffinity assays is the low throughput, which can be improved by optimizing the cell-surface interactions. Micropillars [128] can be used to increase the capture surface area and a herringbone design [129] increases the interactions between the cells and the greater surface area containing capture antibodies via the creation of microvortices.

### **1.6.3 Point-of-care diagnostics**

Point-of-care (POC) testing is done close to the patient and needs to be easy to use, cheap, fast, portable and require small sample volumes [130]. The paper-based lateral flow assay used for pregnancy tests are a very common commercially available POC test, while examples of other such tests for detection of small molecules in blood are glucose measurements for diabetics [131] and measurements of blood gases and electrolytes used in e.g. emergency rooms to improve clinical outcomes [132]. Detection of proteins in blood such as HIV antibodies for HIV diagnostics or presence of *Plasmodium falciparum* for malaria diagnostics are other existing applications for POC tests [133]. Increasing numbers of POC diagnostic tests are being developed for smart phone readouts to further improve the availability and make them easier to use [133, 134]. Microfluidics and lab-on-chip technology can be used to develop cheap and fast POC tests that require small sample volumes.

#### **1.6.3.1 Microfluidics-based blood diagnostic applications**

Microfluidic diagnostic tests are highly researched and show promise for detection of a variety of diseases. As mentioned earlier, immunoaffinity assays and other techniques are being evaluated for both CTC detection in cancer and CD4<sup>+</sup> T cell counts for HIV testing and diagnostics. The concentration tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) in human serum has been analyzed using an electrochemical immunosensor, since it is a biomarker for e.g. inflammatory bowel disease and rheumatoid arthritis [135]. In sepsis diagnostics, there are for example microfluidic chips for both IL-6 quantification in serum [136] and CD64<sup>+</sup> cell capture using a herringbone structure [137], since both are known biomarkers. A microfluidic chip has been designed to capture and detect antibodies towards Lyme antigen in serum from patients with Lyme disease [138]. Microfluidics are being used to further develop and improve a wide range of diagnostic tests including allergy diagnostics.

##### **1.6.3.1.1 Microfluidic applications for allergy diagnostics**

The most common allergy test developed using microfluidics are allergen-specific IgE-ab tests, where the focus has been on miniaturizing and multiplexing the test to enable detection of several allergen-specific IgE-abs simultaneously and quickly. In a microfluidic ELISA, up to 20 allergens can be detected in serum from allergic patients, with a chemiluminescent detection signal [139]. Detection of allergen-specific IgE in serum has also been done using an automated and integrated chip with antigen coating the surface and direct fluorescent labelling [140]. Magnetic core-shell nanoparticles has been coated with the allergen  $\alpha$ -lactalbumin and used to capture allergen-specific IgE-abs from patient serum before detection in a microfluidic chip [141].

There are also microfluidic assays for detection of degranulation of basophils. Most studies are done on different basophil cell lines, and therefore needs to be further validated using human basophils. Lab-on-a-disc (LOAD) is a CD-based platform, which uses centrifugal forces to separate or mix a sample without the need for pumps. In one study, LOAD was used to look at degranulation of human KU812 cell line, which were stimulated with fMLP and house dust mite protein (Der p1) and the released mediators analyzed using acridine orange that fluorescently stains the content of the granules and enables detection of degranulation [142]. In another study, LOAD was coated with conductive transparent indium tin oxide, which can be used to control the temperature. Human KU812 cell line was stained with acridine orange and degranulation after fMLP activation was detected following incubation with an allergy-blocking agent, triptolide [143]. A chip with flow cytometry properties analyzed rat basophilic leukemia cell line (RBL-2H3), and was used to look at single cell FcεRI signaling [144]. RBL-2H3 cell line has also been used to study the effect of histamine release on a monolayer of endothelial cells in a microfluidic chip [145]. Surface plasmon resonance (SPR), is a technique that can detect label-free, real-time changes on a surface using the variation in refractive index that occurs from, e.g. degranulation of captured basophils. SPR can be done in a microfluidic chip, and degranulation of purified human basophils from blood has been studied during exposure to various allergens [146]. However, the detection area is quite small which reduces the throughput. Degranulation can release proteoglycans, which gives the cell a negatively charged surface. This negative charge can be used to detect degranulation using impedance measurements in a microfluidic chip. The negative charge was detected using fluorescently labelled avidin, which binds to strong negative charge and has been used to detect degranulation of rat basophil cell line after incubation with patient serum followed by antigen stimulation [147].

There is still research to be done in the fields of allergy diagnostics and microfluidics, to miniaturize and increase both throughput and availability of allergen-specific IgE-ab tests but especially cellular tests such as BAT.



## 2 AIMS OF THE THESIS

The aims were to improve allergy diagnostics using microfluidic techniques, specifically to develop a microfluidic platform for the basophil activation test (BAT), and to further investigate basophil adhesion and activation.

Specific aims:

**Paper I:** Develop a microfluidic chip that can capture and activate basophils from whole blood.

**Paper II:** Determine if basophils captured in a microfluidic chip can be activated in a dose-dependent manner using allergens and compare the method to flow cytometry-based BAT.

**Paper III:** Study basophil adhesion and activation to better understand human basophil responses both in vivo and in vitro in a microfluidic chip.





### **3 MATERIAL AND METHODS**

In this section, the study populations and material and methods used in the studies are described briefly. More detailed information can be found in each paper.

#### **3.1 Study populations**

The studies were approved by the local Ethics Committee in Stockholm, Sweden, Dnr. 2014/1630-31/4 and 2018/764-31/1. All allergic patients and healthy controls gave their oral and written consent to participate.

##### **3.1.1 Study population in paper I**

Eight allergic patients (6-18 years old) were recruited from Sachs's Children and Youth Hospital, Stockholm, Sweden. The patients were allergic to various airborne and food allergens. Eight healthy controls (18-65 years old) were recruited from the Blood Banks in Stockholm.

##### **3.1.2 Study population in paper II**

Ten allergic patients (6-18 years old) were recruited from Sachs's Children and Youth Hospital, Stockholm, Sweden. The patients were allergic to airborne allergens, i.e. birch, timothy, cat, dog or/and horse, but not allergic to the control allergens house dust mite or bee. Ten healthy controls (18-65 years old) were recruited from the Blood Banks in Stockholm.

##### **3.1.3 Study population in paper III**

Healthy controls (18-65 years old) were recruited from the Blood Banks in Stockholm.

##### **3.1.4 Study population for preliminary results**

Healthy controls (18-65 years old) were recruited from the Blood Banks in Stockholm. Cow's milk allergic children (2-17 years old) recruited from Sachs's Children and Youth Hospital, Stockholm, Sweden.

#### **3.2 Human basophil cell line (KU812) culture**

In paper I and preliminary results, human basophil cell line (KU812) was used. The cells were cultured in RPMI (GE Healthcare Life Sciences, USA) medium containing 10% fetal bovine serum, 0.5% sodium pyruvate solution (Sigma-Aldrich, USA) and 0.2% non-essential amino acids (Sigma-Aldrich). The cells were incubated at 37°C in a CO<sub>2</sub> incubator and the medium was renewed every 3-4 days through standard cell culture practice.

#### **3.3 Blood sampling**

In all papers and preliminary results, venous blood samples were collected in sodium-heparin vacutainer tubes (Vacutainer, Becton Dickinson, USA) and stored at 4°C until analysis. In paper II, serum was collected and frozen at -20°C pending analysis of allergen-specific IgE-abs.

#### **3.4 Methods for basophil analysis**

##### **3.4.1 Flow cytometric basophil activation test (BAT)**

In paper I, blood was incubated with anti-FcεRI (Bülmann Laboratories AG, Switzerland) as a positive control and RPMI (GE Healthcare Life Sciences) as a negative control. The MFI for CD203c and percent positive CD63 (%CD63+) basophils were identified using anti-CD203c-

PE and anti-CD63-FITC (Beckman coulter, Paris, France) and analyzed using flow cytometry (Navios, Beckman Coulter, USA).

In paper II, the blood was incubated with relevant airborne allergens (birch, timothy, cat, dog or horse) and non-relevant airborne allergens (house dust mite or bee) at 5000, 500, 50 and 5 SQU/ml (Aquagen, ALK, Denmark). In addition, anti-FcεRI (Bühlmann Laboratories AG) was used as a positive control and IL-3 and calcium containing stimulation buffer (Bühlmann Laboratories AG) as a negative control. CD203c and CD63 were identified with anti-CD203c-PE and anti-CD63-FITC, and activation determined as %CD63+ basophils. Analysis was done using flow cytometry (Navios, Beckman Coulter).

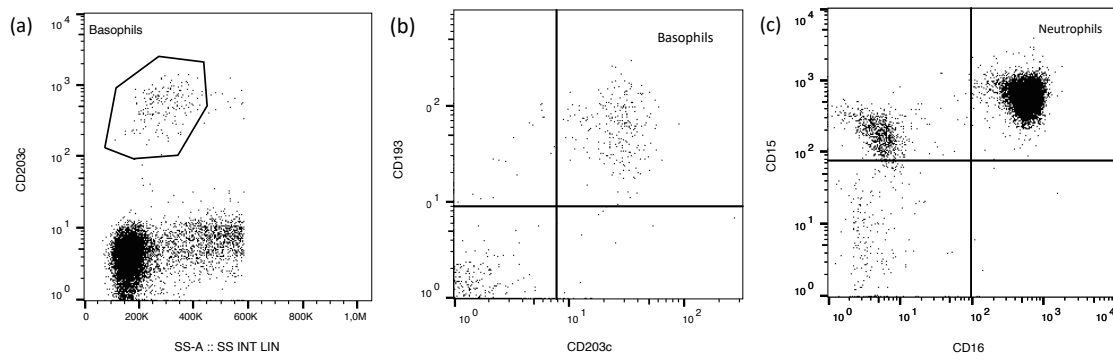
### **3.4.2 Flow cytometric analysis of adhesion molecules and activation markers on basophils**

In paper III, the change in expression ( $\Delta$ MFI) of surface markers after different stimuli was measured on basophils and neutrophils. MFI for CD203c and %CD63+ basophils were analyzed after crosslinking of CD62L (Abcam), CD11b (BioLegend, USA), CD203c (Abcam) or CD49d (Abcam). The %CD63+ basophils and MFI for CD203c were also detected after crosslinking of CD62L, CD11b (Abcam) followed by crosslinking of CD203c (Abcam) or after stimulation with IL-3 (Sigma-Aldrich), IL-33 (ThermoFisher Scientific, USA) or IL-8 (R&D systems, USA) followed by crosslinking of CD203c (Abcam). The effect of IL-3, IL-33 and IL-8 stimulation was determined by measuring the %CD63+ basophils and the  $\Delta$ MFI for CD203, CD62L, CD11b and CD49d on basophils and the  $\Delta$ MFI for CD62L, CD11b and CD49d on neutrophils compared to a control (RPMI). MFI for CD203c and %CD63+ basophils were analyzed after either crosslinking of CD62L, CD11b or CD49d (Abcam) or cytokine stimulation with either IL-3 or IL-33 followed by activation with anti-IgE antibody (Beckman Coulter) or fMLP (Sigma-Aldrich), with RPMI (GE Healthcare Life Sciences) as control. All analysis was done using flow cytometry (Navios, Beckman Coulter, USA).

### **3.4.3 Flow cytometric gating strategies**

In paper I and II, the basophils were gated as CD203c+ (Figure 9a) and activated basophils were gated as CD63+. A cut off for a positive basophil activation test was set to a baseline of 5% (%CD63).

In paper III, the gating strategies were CD203c+ and CD193+ for basophils (Figure 9b) and CD15+ and CD16+ for neutrophils (Figure 9c). MFI was used to evaluate activation of basophils and neutrophils by expression of CD62L, CD11b and CD49d. In addition, basophil activation was also measured as MFI for CD203c and %CD63+. A cut off for a negative test was set to a baseline CD63-expression of approximately 2.5%.



**Figure 9.** Flow cytometry gating strategy for (a) basophils: CD203c+, for (b) basophils: CD193+ and CD203c+ and for (c) neutrophils: CD15+ and CD16+.

### 3.4.4 Serological analyses

In paper II, the serum collected from all allergic patients and healthy controls were analyzed at the Karolinska University Laboratory. IgE-ab to birch, timothy, cat, dog, horse, bee, and dust mite were analyzed using ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden), according to the manufacturer's instructions. The cut off for positive test was set to  $\geq 0.35$  kUA/l.

### 3.4.5 Microfluidic chip design, fabrication, and surface modifications

In paper I and II, a microfluidic chip was designed as a straight channel using AutoCAD with the dimensions for width, length, and height: 4 mm, 25 mm and 50  $\mu\text{m}$ , respectively. The design was printed as a photomask, a film with transparent patterns, which is then used to transfer the pattern onto a master or substrate. A master was made using a silicon substrate by spin-coating with a light sensitive material called a photoresist that can be crosslinked to the surface of the substrate using the photomask and UV light, where non-crosslinked photoresist can be easily removed. A negative photoresist was used (SU-8), where the areas that are exposed to UV light becomes insoluble and can then be used as a mold for creating microfluidic chips. Polydimethylsiloxane (PDMS) is an elastomer commonly used to create microfluidic chips for biological applications and is a good choice based on its properties involving transparency, biocompatibility and gas-permeability through the elastomer, all of which are important when working with cells. PDMS was poured onto the master and cured at 65°C for 6 hours, before the channels could be cut and peeled from the master. The holes for inlets and outlets were punched using a biopsy puncher (0.75mm) before the PDMS was bonded to a glass slides using an oxygene plasma chamber.

In preliminary results, a microfluidic chip was designed using AutoCAD with 16 parallel straight channels, with the same dimensions per channel as the design in paper I and II (width: 4 mm, length: 25 mm and height: 50  $\mu\text{m}$ ). The design had one inlet and eight outlets. The master and microfluidic chips were made the same way as in paper I and II.

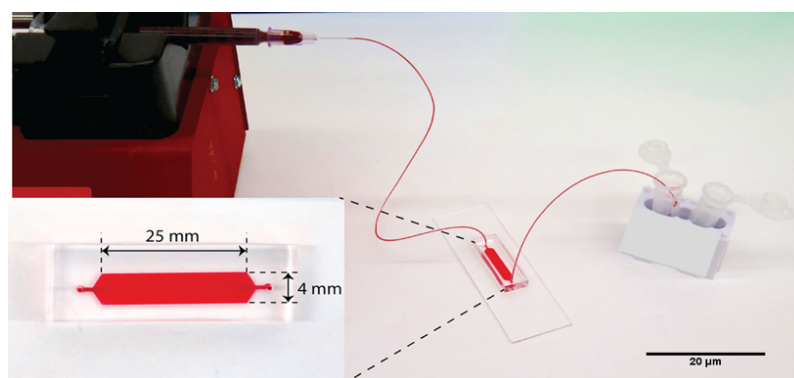
The walls inside of the microfluidic chips are hydrophobic, so the chips were treated with 3-mercaptopropyl trimethoxysilane chemistry (Sigma-Aldrich) followed by the linker 4-Maleimidobutyric acid N-hydroxysuccinimide ester (GMBS) (Sigma-Aldrich) and NeutrAvidin® (Sigma-Aldrich). Biotinylated anti-CD203c (basophil specific) antibodies (Miltenyi Biotec, Germany) were then attached to the NeutrAvidin® on the chip surface. The microfluidic chips were stored at 4°C until use.

### 3.4.6 Basophil capture in microfluidic chips

#### 3.4.6.1 Human basophil cell line (KU812)

In paper I, the flow rates for basophil capture and washing was optimized using human basophil cell line (KU812). 1% bovine serum albumin (BSA) was pumped through the channel at a flow rate of 20  $\mu\text{l}/\text{min}$  using a syringe pump (Harvard apparatus, USA) followed by KU812 cell line diluted in RPMI (Thermo Fisher Scientific) flowing at 1-20  $\mu\text{l}/\text{min}$ . The unbound cells were then washed out at 20  $\mu\text{l}/\text{min}$  using 1% BSA and the captured cells were stained using Hoechst stain (Sigma-Aldrich). The captured cells in the chip were then imaged using a Nikon Ti Eclipse fluorescent microscope (Nikon, Tokyo, Japan). The experimental set up can be seen in Figure 10.

In preliminary results, the multiple parallel channel chip was also optimized using KU812 cell line in RPMI (Thermo Fisher Scientific) at 50, 60 and 70  $\mu\text{l}/\text{min}$  after blocking with 1% BSA. The unbound cells were washed out at 320  $\mu\text{l}/\text{min}$ , captured cells were stained using Hoechst stain (Sigma-Aldrich) and the chip was imaged using fluorescent microscopy (Nikon). Microfluidic chips were also made with and without capture antibody (anti-CD203c) (Abcam) and the number of captured KU812 cell line cells were imaged and then counted using CellProfiler.



**Figure 10.** Experimental set up with a syringe pump, where the syringe is connected to a microfluidic chip. In the bottom left hand corner is the design and dimensions of the single channel chip.

#### 3.4.6.2 Whole blood

In paper I, basophils from whole blood was captured in the microfluidic chip at different flow rates (3-10  $\mu\text{l}/\text{min}$ ) followed by washing with 1% BSA at 20  $\mu\text{l}/\text{min}$ . The captured cells were then stained with Hoechst stain and fluorescently labeled with primary anti-CD203c antibody followed by secondary anti-mouse antibody conjugated to PE (Abcam).

In the activation experiments, CD63 was also fluorescently labeled with anti-CD63-Alexa647 (Abcam). Imaging was done using fluorescent microscopy (Nikon). The number of basophils (CD203c+) were detected before and after capture on chip, using flow cytometry analysis (Navios). The number of CD203c+ (basophils) and CD203c+, CD63+ (activated basophils) cells were counted to get the %CD63+ basophils. The optimal flow rates were then used in all experiments in paper I and II for capture of basophils from whole blood.

In paper II, the cells were captured, labeled and detected in the same way as in paper I except CD203c was stained with a different secondary antibody, anti-mouse-Alexa488 (Abcam). The number of %CD63+ basophils were counted.

In preliminary results, the parallel channel chips were prepared with and without capture antibody (anti-CD203c) (Abcam, UK) and whole blood was pumped at 50  $\mu$ l/min followed by washing at 320  $\mu$ l/min. The number of cells captured were imaged after Hoechst staining and the total number of captured cells counted using CellProfiler.

### **3.4.7 Flow cytometric analysis of surface markers after flowing through a microfluidic chip**

Whole blood from three healthy donors was pumped through six straight channel chips blocked with 1%BSA without capture antibody (anti-CD203c) at 3  $\mu$ l/min. The blood was collected at the outlets. %CD63<sup>+</sup> basophils, MFI for CD203c, CD62L, CD11b and CD49d (BioLegend) on basophils and MFI for CD62L and CD11b (Biolegend) on neutrophils were compared before and after flowing through the microfluidic chip using flow cytometry (Navios).

### **3.4.8 Basophil activation in the microfluidic chip**

#### **3.4.8.1 *Anti-Fc $\epsilon$ RI***

In paper I, the captured basophils from whole blood donated by allergic patients and healthy controls were activated using anti-Fc $\epsilon$ RI (Bühlmann Laboratories) as a positive control and RPMI (GE Healthcare) as a negative control. The captured cells were fluorescently labeled with mouse anti-CD203c and anti-mouse-PE (Abcam) and anti-CD63-Alexa647 (Abcam) before imaging using fluorescent microscopy (Nikon). The number of %CD63<sup>+</sup> basophils were counted.

#### **3.4.8.2 *Airborne allergens***

In paper II, basophils from allergic patients were activated using airborne allergens (birch, timothy, cat, dog or horse) at two different concentrations (5000 and 50 SQU/ml). One of the non-relevant control allergens (dust mite or bee) was also analyzed at the same concentrations. As a positive control anti-Fc $\epsilon$ RI (Bühlmann Laboratories) was used and as a negative control stimulation buffer (Bühlmann Laboratories) was used. The captured cells were fluorescently labeled with mouse anti-CD203c and anti-mouse-Alexa488 (Abcam) and anti-CD63-Alexa647 (Abcam) before imaging using fluorescent microscopy (Nikon). The number of %CD63<sup>+</sup> basophils were counted.

### **3.5 Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5 and 7.0e (GraphPad Software, USA). Mann-Whitney U test for two independent sample groups (paper I and II) was applied to analyze non-normally distributed values. The Bland-Altman method was used to compare the data from the two tests (BAT and miBAT) in paper II. In paper III, Wilcoxon test with paired t-test was used for non-parametric values. Correlations in paper III were measured using the two-tailed non-parametric Spearman correlation test. A p value of <0.05 was considered significant.



## 4 RESULTS AND DISCUSSION

### 4.1 Microfluidic immunoaffinity basophil activation test for point-of-care allergy diagnosis (Paper I)

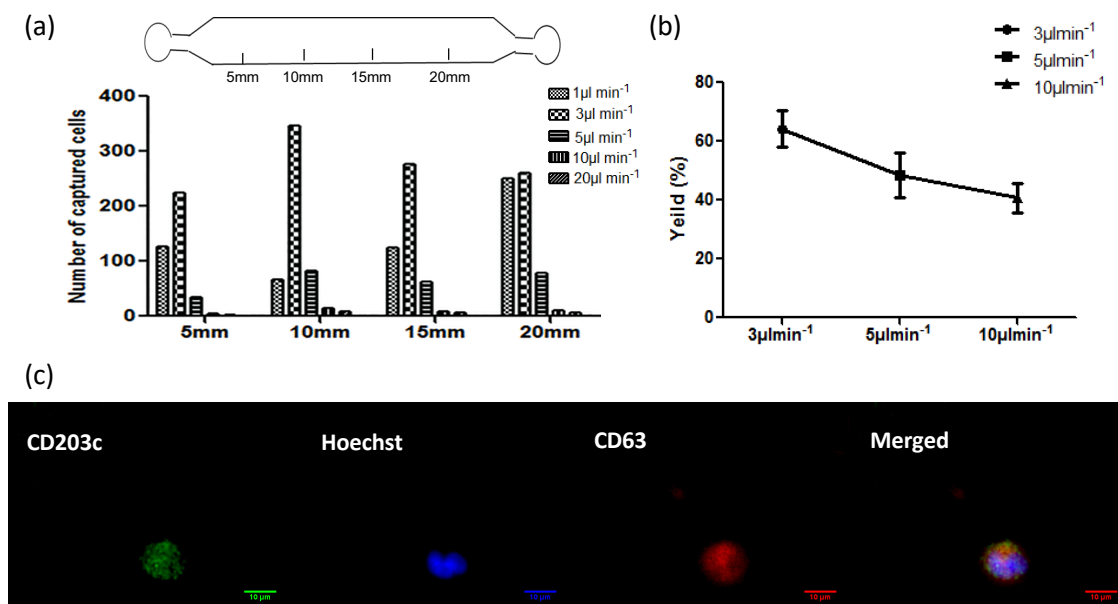
The basophil activation test (BAT) is an in vitro diagnostic allergy test that mimics an in vivo challenge but is performed in a test tube, therefore eliminating any risk of adverse reactions from physical contact with an allergen. In BAT, basophils in blood are stimulated with allergens and if the allergen crosslinks IgE-abs on the surface of the cell, it will cause the cell to degranulate and release e.g. histamine. Degranulation will also cause the basophil to express activation markers such as CD63 and CD203c on the surface of the cell, which can then be analyzed using flow cytometry [81]. However, BAT is mainly available at university hospitals at present due to the need for an expensive flow cytometer, which also requires skilled personnel to operate. To improve the availability and reduce the cost of BAT we have in paper I developed a microfluidic immunoaffinity BAT (miBAT) that is analyzed using microscopy.

The microfluidic chip was designed as a straight channel (width: 4 mm, length: 25 mm, height: 50  $\mu$ m). The optimal shear stress for basophil (KU812) cell line capture using an anti-CD203c antibody attached to the chip walls was determined using a Hele-Shaw chip. In the Hele-Shaw device the shear stress decreases along the length of the chip due to the increased width and allows for optimization of shear stress for a specific target cell and capture antibody [122]. The optimal cell capture flow rate, derived from the optimal shear stress, of the straight channel chip design corresponded to 3  $\mu$ l/min, which could be confirmed as the optimal capture flow rate (Figure 11a). The optimal washing flow rate was determined to 20  $\mu$ l/min.

Using whole blood, the optimal yield of captured basophils was 64% (Figure 11b) at 3  $\mu$ l/min and the purity of captured basophils was 40% (number of captured basophils divided by total number of captured leukocytes). The captured cells from blood in the microfluid chip were stained with Hoechst stain (nucleus) and anti-CD203c and anti-CD63 fluorescently labeled antibodies (Figure 11c). The nuclear stain was used for identification of cells, CD203c is a basophil-specific surface marker used for identification [41], and CD63 is expressed on the surface of basophils after anaphylactic degranulation and therefore used for identification of activated basophils in combination with CD203c [41].

After optimization of basophil capture from blood, stimulation of the captured basophils was done using a positive (anti-Fc $\epsilon$ RI) and a negative (RPMI) control to determine whether it was possible to activate the basophils after capture in a microfluidic chip. The percentage of CD63-positive (%CD63+) basophils and the MFI for CD203c were compared between eight healthy donors and eight allergic patients as well as between BAT and miBAT. There was an upregulation in %CD63+ basophils after anti-Fc $\epsilon$ RI activation in the microfluidic chip for both healthy controls and allergic patients. The difference in %CD63-expression between positive and negative controls were significant both in miBAT ( $p=0.0002$ ) and BAT ( $p=0.0009$ ) (Figure 12a). We also observed a significant difference in %CD63 expression between allergic donors and healthy controls using both BAT ( $p=0.03$ ) and miBAT ( $p=0.04$ ). The difference in %CD63-expression between allergic patients and healthy donors opens up the possibility of miBAT-based allergy diagnostics. Basophils from allergic patients have a tendency to express higher levels of CD63 after activation than healthy donors [148]. It has also been reported that

atopic patients have higher expression levels of FcεRI than non-atopic donors [149] and that IgE levels generally are higher in allergic patients than healthy controls [78]. However, the test would be more relevant if allergen stimulation was analyzed instead of positive and negative controls only since this would give information on the individual allergy profile. There was no significant difference in CD63-expression between miBAT and BAT in allergic patients, indicating that the tests gave comparable results.



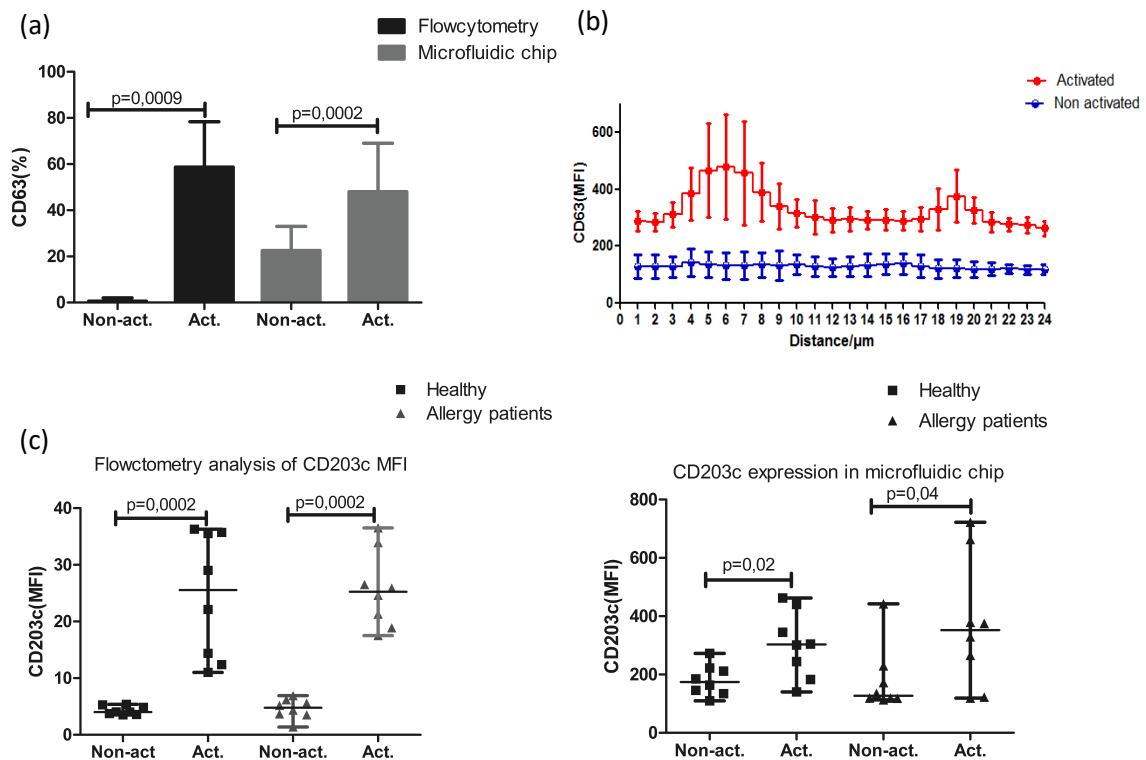
**Figure 11.** Basophil flow rate optimization and basophil imaging using fluorescent microscopy. (a) Characterization of the microfluidic chip using basophil cell line (KU812) at different capture flow rates. (b) Yield (%) of captured basophil from blood at different capture flow rates,  $n=3$ . (c) Fluorescent staining of a basophil captured from blood in a microfluidic chip: CD203c, Hoechst (nuclear stain), CD63 and merged. Scale bar: 10  $\mu\text{m}$ .

CD203c is used as an identification marker for basophils as well as an activation marker for both anaphylactic and piecemeal degranulation [41]. MFI for CD203c was therefore detected in both miBAT and BAT after stimulation with anti-FcεRI. In BAT the upregulation of MFI for CD203c after basophil activation (anti-FcεRI) was significant for both healthy donors ( $p=0.0002$ ) and allergic patients ( $p=0.0002$ ) (Figure 12c). The same observation was made for miBAT for both healthy donors ( $p=0.02$ ) and allergic patients ( $p=0.04$ ) (Figure 12c), allowing for the possibility to use CD203c as an activation marker for miBAT, alone or in combination with CD63. CD63 is specific for anaphylactic degranulation caused by e.g. IgE crosslinking whereas CD203c is also upregulated by other types of stimulation, such as cytokines like IL-3 [150]. MFI for CD203c could therefore be of interest when studying other types of basophil activation and functions like piecemeal degranulation and changes in e.g. adhesion molecule expression.

In the microfluidic chip there is a background activation (%CD63) in the negative control, which does not result in a higher activation in the positive control. The background activation could be due to for example handling of the blood prior to capture, which is why the handling needs to be done carefully by for example keeping the blood at 4°C before use and avoid unnecessary stress on the cells, or possibly due to contamination from e.g. pyrogens and endotoxins from bacteria during chip fabrication, which is why it is important to assemble the



chips in a sterile environment. The material in the microfluidic chip can also be the cause since even though PDMS is a biocompatible polymer it has been shown to activate neutrophils [151]. The background activation could also be due to the shear stress [152] from the friction between the fluid from example the washing steps that works on the captured basophils in the chip or alternatively primes the cells for activation at a later stage. Another factor that might affect the background activation is the processing time before fixation, which can be minimized by further optimization of the microfluidic chip to e.g. increase the capture flow rate and/or increase the capture efficiency to minimize the time required for basophil capture. A herringbone structure has been tested to determine if this could reduce the processing time by increasing the interactions between the basophils and the capture antibody. However, this did not improve the capture efficiency. There was no difference in background activation between allergic patients and healthy controls and varied more between individuals. In addition, we measured the MFI for CD63 on activated basophils, both for a positive and a negative control and compared the results. The MFI for CD63 was higher in activated basophils in the positive control compared to unstimulated basophils in the negative control (Figure 12b), meaning that this could potentially be used as a threshold for image analysis. A threshold would be set to distinguish between real and background activation (MFI CD63) and count only the signals above the threshold as activated basophils.



**Figure 12.** Microfluidic chip and flow cytometry analysis of basophils from blood stimulated with controls. (a) %CD63+ basophils from blood captured in a microfluidic chip or analyzed using flow cytometry after stimulation with RPMI (non-activated) and anti-Fc $\epsilon$ RI (activated). (b) Measurements of MFI CD63 ( $n=3$ ) over the length of captured basophils from blood in a microfluidic chip, stimulated with either RPMI (non-activated) or anti-Fc $\epsilon$ RI (activated). (c) Flow cytometry and microfluidic chip analysis of MFI CD203c after basophil stimulation with anti-Fc $\epsilon$ RI (activated) and RPMI (non-activated) using blood from healthy donors ( $n=8$ ) and allergic patients ( $n=8$ ). A  $p$ -value of  $<0.05$  was considered significant.

To our knowledge, there are at present no other microfluidic techniques capable of capturing and stimulating human basophils from whole blood. Most microfluidic assays are developed using basophil cell line, such as the CD-based platform lab-on-a-disc (LOAD) [142, 143] and the impedance measurements of the negative charge from degranulation in a microfluidic chip [147]. Surface plasmon resonance (SPR), detects changes in refractive index in captured cells and has been used to study the degranulation of captured human basophils from blood, but after prior purification steps [146].

In paper I, we showed that the miBAT can be used to capture and activate human basophils from whole blood. The test can significantly distinguish between negative and positive controls and between healthy donors and allergic patients. miBAT and BAT give comparable results.

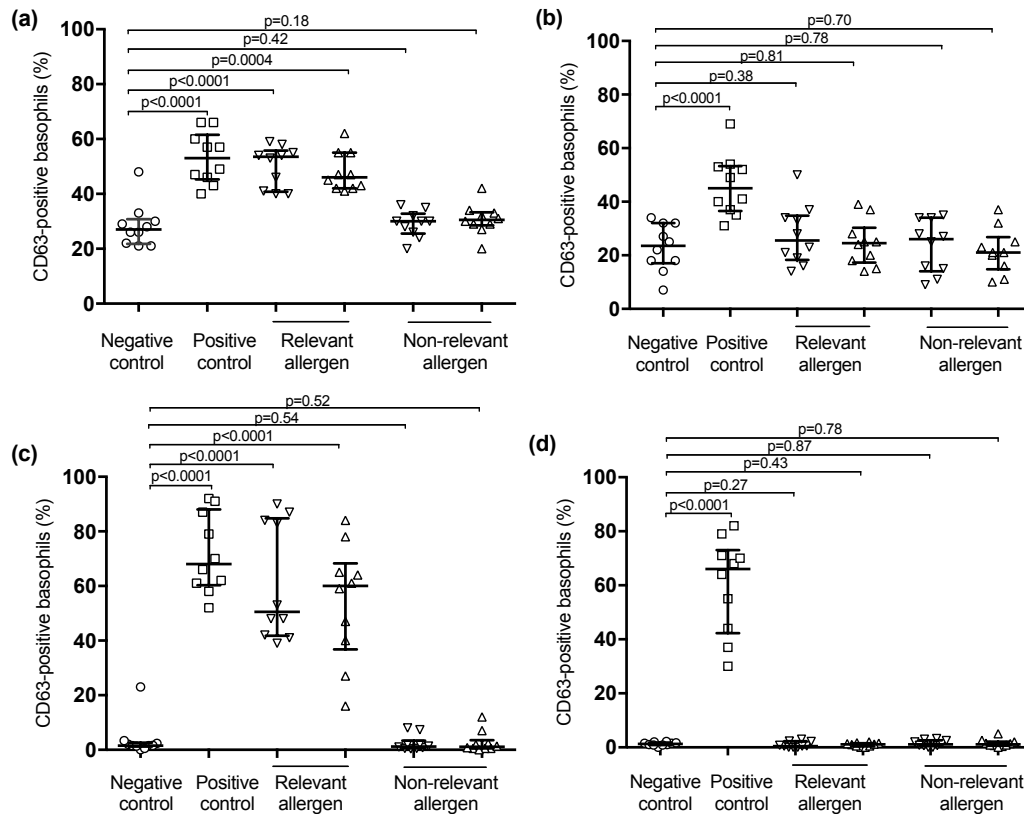
## **4.2 A novel tool for clinical diagnosis of allergy operating a Microfluidic Immunoaffinity Basophil Activation Test Technique (Paper II)**

Due to the promising results in paper I, we continued to evaluate the microfluidic immunoaffinity BAT (miBAT) for allergy diagnostics using allergens in paper II. The basophils captured in the miBAT were stimulated with an airborne allergen (birch, timothy, cat, dog or horse) and a control allergen (dust mite or bee) at two concentrations each (50 and 5000 SQU/ml) as well as with anti-Fc $\epsilon$ RI (positive control) and stimulation buffer (negative control). We recruited blood samples from 10 allergic individuals and 10 healthy controls. The allergic individuals were sensitized to at least one of the airborne allergens (relevant allergens), but not towards the control allergens (non-relevant allergens).

The percentage of CD63-positive (%CD63+) basophils were analyzed using the flow cytometry-based basophil activation test (BAT) and miBAT in parallel. In miBAT the relevant allergens at 50 and 5000 SQU/ml ( $p < 0.0001$ ,  $p = 0.0004$ , respectively) and the positive control ( $p < 0.0001$ ) showed a significantly higher activation than the negative control. The non-relevant allergens did not show a significant activation in CD63-upregulation compared to the negative control in allergic patients (Figure 13a). In healthy controls, stimulation with anti-Fc $\epsilon$ RI (positive control) ( $p < 0.0001$ ) resulted in significantly more activated basophils compared to stimulation buffer (negative control), but no significant difference was observed between the negative control and the relevant or non-relevant allergens (Figure 13b). When analyzing the %CD63+ basophils using BAT, the positive control ( $p < 0.0001$ ) and relevant allergens at 50 and 5000 SQU/ml ( $p < 0.0001$  for both), were significantly higher than the negative control in allergic patients. There was no significant difference between stimulation with non-relevant allergens and stimulation buffer (negative control) (Figure 13c). In healthy controls the %CD63-expression for the negative control was only significantly lower than the positive control ( $p < 0.0001$ ) (Figure 13d).

The high background activation remains at a median of 26% (21-31.5%), and to improve the miBAT technique further this needs to be addressed. One solution would be to use a threshold for image analysis using MFI CD63 as discussed in paper I. This could be combined with an automatic image analysis program for cell identification (nuclear stain) followed by basophil identification (CD203c+) and %CD63+ basophil calculations. Examples of programs that could be used are CellProfiler and ImageJ.

To compare miBAT to BAT, we used the Bland-Altman test. However, since BAT is performed using whole blood and the captured basophils in miBAT are stimulated after capture and washing (purification) the results will not be quite comparable. It has been shown that BAT performed in whole blood does not give the exact same dose-response curve as in washed blood (without plasma) [153], possibly due to factors found in plasma. Factors such as allergen-specific IgG4, which can block allergen binding to IgE [77] or bind to the inhibitory IgG receptor (CD32b) blocking IgE-mediated activation [44]. A way to address this would be to remove the plasma from the blood in BAT analysis. Another consideration is the cells that are still present in blood during BAT stimulation contrary to after capture in miBAT, where possible other factors such as cell interactions or cytokine secretion could affect the basophil activation. Therefore, another alternative would be to purify the basophils before BAT, using e.g. a density gradient which isolates basophils together with other cells with similar density that can then be further purified by negative selection using antibody-coated magnetic beads for further enrichment [154]. Alternatively, dilute the allergens in plasma from the allergic patient before capture in the miBAT to include all factors in plasma that can have an effect on basophil degranulation [155].



**Figure 13.** Difference in CD63-expression on activated basophils compared to non-activated basophils ( $n=10$ ). CD63-expression on basophils stimulated with a negative control (stimulation buffer), positive control (anti-Fc $\epsilon$ RI) and allergen (50 SQU/ml ( $\nabla$ ) and 5000 SQU/ml ( $\Delta$ )) (a) captured in a microfluidic chip from allergic donors, (b) captured in a microfluidic chip from healthy controls, (c) analyzed with flow cytometry-based BAT from allergic patients and (d) analyzed with flow cytometry-based BAT from healthy donors. Results are presented as median and interquartile range. A  $p$ -value of  $<0.05$  was considered significant.

In conclusion, paper II shows that it is possible to activate the captured basophils from allergic patients in miBAT using allergens and to distinguish between activation using relevant and non-relevant allergens.

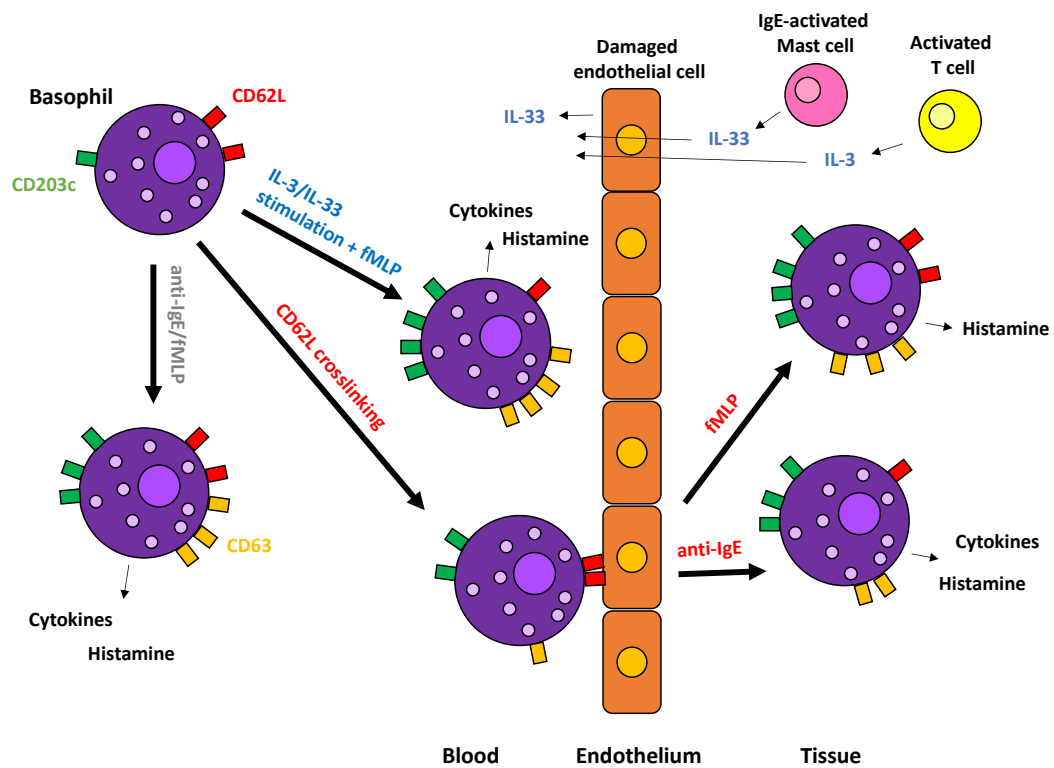
### **4.3 Adhesion molecule crosslinking and cytokine exposure modulate IgE and non-IgE- dependent basophil activation (Paper III)**

Basophils are rare granulocytes circulating in blood and are known for their role in the defense against parasites and in allergic disease but have also been found to have other functions involving both innate and adaptive immunity. To perform its functions, the basophil can transmigrate into tissue. The signaling for recruitment starts with cytokines secreted from cells at the site of the inflammation, from e.g. mast cells [57] or damaged endothelial cells [47]. Different adhesion molecules are then involved in tethering, rolling, adhesion and transmigration [53]. Once in tissue, basophils can secrete IL-4 to drive Th2 immunity, recruit other cells or degranulate and release histamine. Basophil degranulation can occur via both IgE-dependent [30] or IgE-independent pathways [156]. In paper III, we have started to address the background activation seen in the negative controls in paper I and II, where unstimulated basophils captured in a microfluidic immunoaffinity BAT (miBAT) chip shows an upregulation in %CD63-expression. We have also studied changes in activation marker expression (CD63 and CD203c) after crosslinking of adhesion molecules, cytokine stimulation and activation to investigate how the basophils react to stimuli in a microfluidic chip and also to study basophil response while mimicking parts of in vivo processes and stimuli (Figure 14).

To better understand the cause of the background activation seen in miBAT we investigated whether handling and the passage through the microfluidic chip influenced surface marker expression. It has been reported in other studies that neutrophils are activated when in presence of PDMS [151] and since basophils belongs to the granulocyte family, the up -or downregulation of several surface markers were of interest to study. The basophil activation markers included CD203c and CD63 while the adhesion molecules included CD62L, CD11b and CD49d for both basophils and neutrophils. The expressions were studied before and after passage through the microfluidic chip, which did not contain capture antibody (anti-CD203c). For neutrophils, the expected significant shedding of CD62L ( $p=0.0312$ ) could be confirmed. For basophils, CD203c, CD62L, CD11b and CD49d had a quite stable expression, with a slightly but significantly upregulation in MFI ( $p=0.0312$  for all). The %CD63-expression on basophils was not upregulated but instead had a very slight but significant downregulation when comparing before and after handling and flow through the microfluidic chip (Figure 15a). These steps could therefore not explain the background activation. However, the upregulation of MFI for CD203c and CD11b does indicate some form of activation or priming of the basophil for adhesion. CD203c and CD11b have for example been shown to be upregulated simultaneously [157] upon activation as well as by cytokines such as by IL-3 [158] and IL-33 [49], which might be present in plasma.

Next, we investigated whether crosslinking of different surface markers expressed on basophils caused an upregulation in %CD63-expression. Crosslinking mimics capture in the microfluidic chip but also adhesion to the endothelium in vivo (Figure 14). CD203c is used for basophil-specific capture in the microfluidic chip where anti-CD203c antibodies are

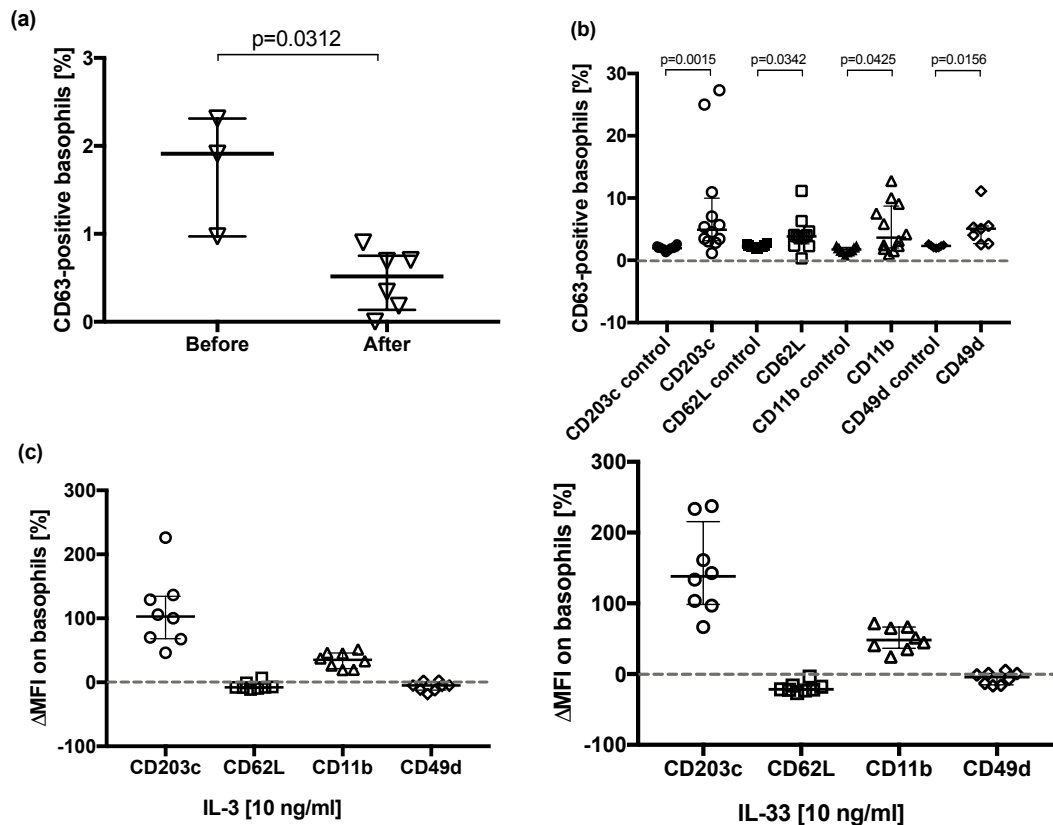
attached to the surface of the chip. Crosslinking of the adhesion molecules CD62L, CD11b and CD49d were also studied since they are adhesion molecules and might also interact with the surface of the chip or proteins adsorbed from blood by PDMS. PDMS is known to adsorb proteins from blood, e.g. fibronectin [151] that binds CD49d and CD11b [63]. Crosslinking of CD203c, CD62L, CD11b and CD49d caused a significant upregulation of %CD63+ basophils (Figure 15b). The median increase in %CD63-expression after crosslinking was for: CD203c 4.93%, CD62L 3.85%, CD11b 3.67% and CD49d 5.1%. Crosslinking of CD203c might therefore partly explain the background activation seen in miBAT. Crosslinking of adhesion molecules to adsorbed proteins onto PDMS should not occur since the surfaces are blocked using 1% BSA [159]. Although, the blocking might require further improvements to reduce unspecific binding. Considering that the %CD63-expression from surface marker crosslinking is quite low compared to the total background activation seen in miBAT, it indicates that this is a minor factor.



**Figure 14.** Schematic summary of CD63 and CD203c expression after basophil stimulation with IL-3, IL-33, anti-IgE and fMLP as well as crosslinking of CD62L, which mimics possible scenarios involving stimulation and adhesion in vivo.

Since passage through the microfluidic chip activated neutrophils (CD62L shedding) and considering PDMS has been shown to stimulate e.g. macrophages to produce cytokines [151], it is possible that there is an increase in cytokine exposure during the capture phase in the microfluidic chip. Cytokines are crucial for basophil recruitment to tissue in vivo and two such cytokines are IL-3 and IL-33. IL-3 can be secreted by T cells and regulatory T cells during parasite infection [59, 160] and IL-33 is released from e.g. damaged endothelial and epithelial cells [37] and activated mast cells [57], see Figure 14. Both IL-3 and IL-33 significantly upregulated MFI for CD203c ( $p=0.0078$ ,  $p=0.0391$ , respectively) and CD11b ( $p=0.0078$ ,  $p=0.0078$ , respectively) on basophils (Figure 15c), as expected.

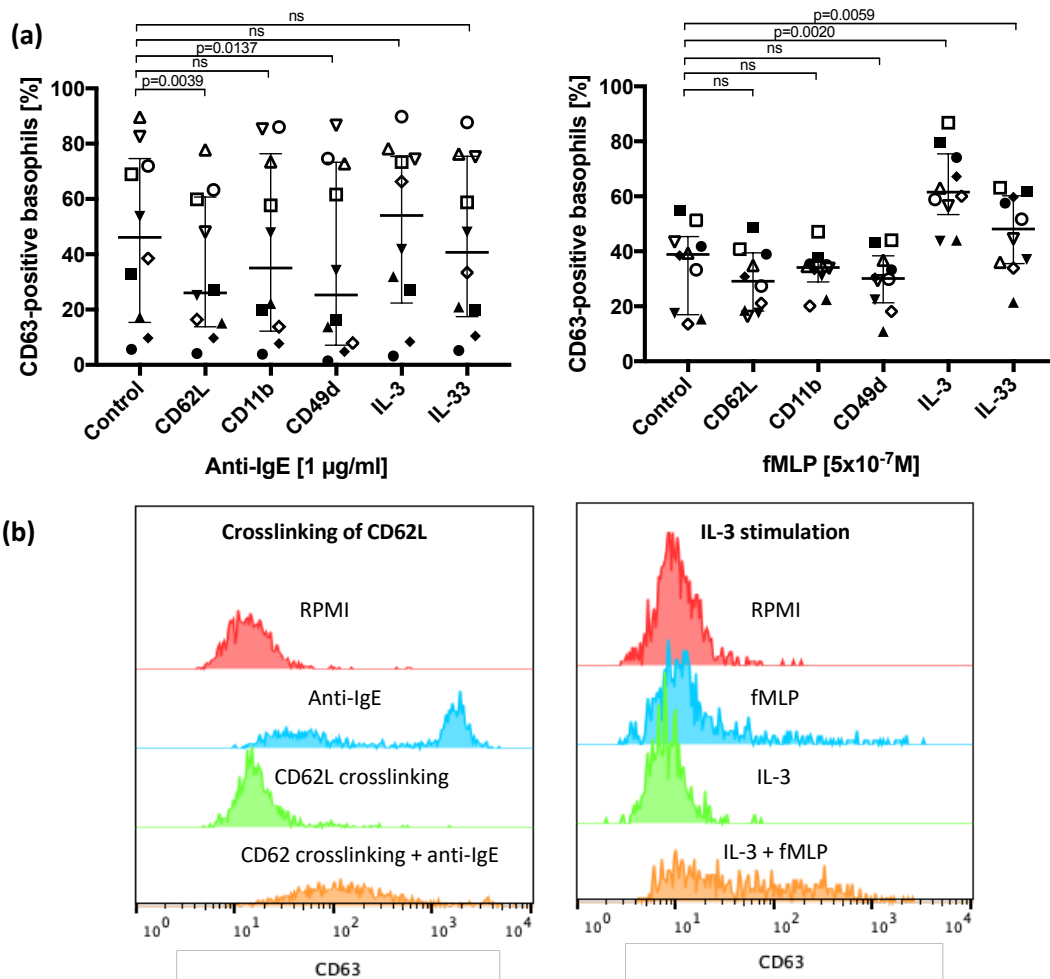
IL-33 (median -21.6%,  $p=0.0078$ ) had a more significant effect on CD62L shedding from basophils than IL-3 (median -7.9%,  $p=0.0469$ ). IL-33 induced CD62L shedding might tune the CD62L binding to ligands in tissue, such as proteoglycans [61]. It has been reported that CD62L is quite stable on basophils upon stimulation via several pathways such as TLR2, TLR4 and fMLP but that anti-Fc $\epsilon$ RI activation significantly downregulates CD62L [60].



**Figure 15.** Surface marker expression on basophils. (a) %CD63+ basophils before and after passage through a microfluidic chip. (b) %CD63+ basophils after crosslinking of CD203c, CD62L, CD11b and CD49d. (c)  $\Delta$ MFI for basophil expression of CD203c, CD62L, CD11b and CD49d after cytokine stimulation using IL-3 and IL-33. Results are presented as median (and interquartile range). A  $p$ -value of  $<0.05$  was considered significant.

Anaphylactic degranulation of basophils instantly releases histamine and cytokines such as IL-4 as well as expresses CD63 on the cell surface in response to activation. Both IgE-dependent (allergen crosslinking) and IgE-independent (fMLP) activation can cause anaphylactic degranulation in basophils [161] and occur in mast cells response to infection or during allergic reactions [39] and therefore likely also in basophils. Piecemeal degranulation is another type of degranulation process that is thought to induce paracrine and endocrine signaling [38] and does not release much histamine or upregulate CD63 on basophils [161]. To better understand adhesion and cytokine stimulation in combination with degranulation, we crosslinked adhesion molecules or stimulated the basophils with cytokines before IgE-dependent or IgE-independent degranulation (Figure 14). Crosslinking of CD62L ( $p=0.0039$ ) and CD49d ( $p=0.0137$ ) significantly reduced IgE-dependent degranulation (anti-IgE antibody) and the %CD63-expression compared to only anti-IgE crosslinking (Figure 16a-b). This has previously been reported for CD49d [162] but to our knowledge not for CD62L. The lower expression of CD63 could indicate a regulatory function appointed to

reduce IgE-mediated degranulation until the cell has transmigrated into tissue or possibly promote cytokine production over degranulation. Basophils have been reported to potentially have dual roles in IgE-mediated inflammation, one at lower allergen concentrations where optimal secretion of cytokines such as IL-4 and IL-13 allow for e.g. Th2 differentiation and B cell class switching and one where the cell degranulates in response to a higher allergen concentration, releasing histamine in for example late phase allergic reactions [163, 164]. Crosslinking of adhesion molecules followed by IgE-dependent activation mimics capture and activation in miBAT. Interestingly, we have noted a lower %CD63-expression in miBAT compared to flow cytometry-based BAT after anti-FcεRI activation. It would therefore be of interest to further study CD203c crosslinking and IgE-dependent degranulation since this would best mimic the capture and activation in miBAT. Due to the reduced IgE-dependent activation in miBAT, it would also be interesting to activate the captured basophils using fMLP since we did not see the same regulatory effect for IgE-independent degranulation.



**Figure 16.** CD63-expression on basophils after adhesion molecule crosslinking or cytokine stimulation followed by activation. (a) %CD63+ basophils after crosslinking of CD62L, CD11b or CD49d or after cytokine stimulation with IL-3 or IL-33 followed by either IgE-dependent (anti-IgE ab) or IgE-independent (fMLP) activation compared to the control (RPMI). (b) Histograms for CD63 expression; RPMI and anti-IgE activation compared to CD62L crosslinking followed by anti-IgE activation. Also, RPMI and fMLP activation compared to IL-3 priming followed by fMLP activation. Results are presented as median and interquartile range. A p-value of <0.05 was considered significant.

IL-3 ( $p=0.002$ ) and IL-33 ( $p=0.0059$ ) priming significantly increased the CD63-expression after fMLP activation compared to fMLP activation alone (Figure 16a-b). This has previously been reported for IL-3 [165] but to our knowledge not for IL-33. IL-33 stimulates basophils and eosinophils in blood and is known to enhance histamine and cytokine release in IgE-dependent basophil activation [166]. Here we show a possible role also in IgE-independent activation using fMLP (Figure 14). FMLP alone does not induce cytokine production after basophil activation but requires IL-3 for cytokine (e.g. IL-4) secretion [167], which could potentially also be the case for IL-33 in combination with fMLP since IL-33 alone promotes IL-4 and IL-13 expression.

We have previously fixed and stained the captured basophils directly after the washing step that follows basophil capture from whole blood in miBAT. We could already at this point detect some background activation. We have also detected an additional increase in background activation after the stimulation and incubation steps, meaning that the background originates from both before activation (capture and washing) but also from the IgE-mediated activation step. The basophil capture (crosslinking of CD203c) seems to be a minor factor, whereas washing likely impacts the background activation due to shear stress that will be active on the captured cells [152]. Using ethylenediaminetetraacetic acid (EDTA) as anti-coagulant instead of sodium heparin might reduce background activation since degranulation is calcium dependent and EDTA has the ability to sequester calcium [168, 169]. This would require additional calcium in the buffer used to dilute the allergen before basophil activation. However, when we tried to capture basophils from blood with EDTA instead of sodium heparin as anti-coagulant there was no basophils captured in the microfluidic chip. This could possibly be due to a lower expression of CD203c, which has been reported when comparing EDTA and sodium heparin [168].

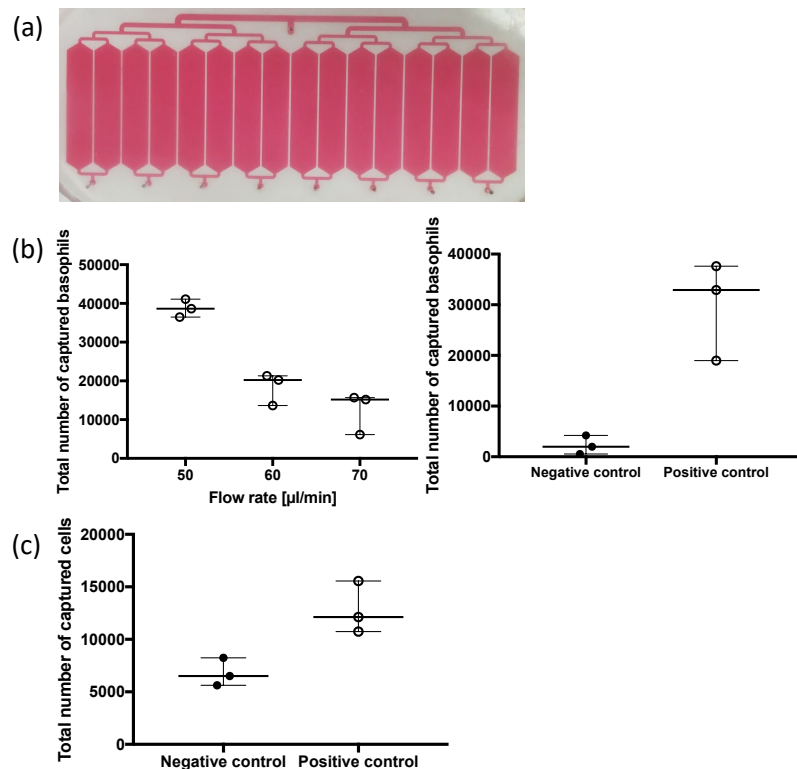
In conclusion, we show that the capture of basophils in miBAT is a potential factor in the background activation seen in the negative controls in paper I and II. Also, crosslinking of CD62L and CD49d have a regulatory effect on IgE-dependent degranulation, which might help explain the lower %CD63-expression in miBAT compared to in flow cytometry-based BAT. In vivo, the regulatory effect of CD62L and CD49d crosslinking could work together with cytokines such as IL-33 that causes CD62L to slightly shed and therefore might affect the regulatory function of IgE-dependent degranulation. More work is needed to better understand how basophils behave both in miBAT and in vivo during adhesion, activation and degranulation.

#### **4.4 Preliminary results: CD-sens on chip**

Allergies affect approximately 40% of children, but some allergies are outgrown within the first few years of life. Food allergies like cow's milk and hen's egg are two examples of common food allergies in children that are often outgrown early [170]. CD-sens is a useful test to measure allergen sensitivity over time in allergic patients [91] to determine when it is safe to perform an oral challenge to verify that the child is no longer allergic and the food can be reintroduced into the diet. We have therefore designed a microfluidic chip to perform CD-sens (CD-sens on chip) on milk allergic children that will be compared to flow cytometric CD-sens.



Several multi-channel chips were designed using AutoCAD and the corresponding masters were produced. The capture and washing steps were optimized using KU812 cell line. The design finally chosen was a multi-channel chip with 16 straight parallel channels and one inlet and eight outlets (Figure 17a), due to the large surface available for basophil capture (twice the area of the single channel chip in paper I and II) and the high number of parallel channels enabling analysis of up to eight allergen concentrations simultaneously in one chip or six allergen concentrations and two controls (positive and negative). Via slight adjustments to the design by adding outlets to each channel, up to 16 different allergen concentrations can be analyzed simultaneously. The optimal flow rates for capture of basophils and washing was determined to 50  $\mu\text{l}/\text{min}$  and 320  $\mu\text{l}/\text{min}$ , respectively using KU812 cell line (Figure 17b). The flow rates correspond well to the optimal flow rates used in the straight channel chip used in paper I and II, but multiplied with 16, since the dimensions are the same for each channel. The optimal flow rates were also used to capture basophil from whole blood in microfluidic chips with and without capture antibody (anti-CD203c) (Figure 17c) and the total number of captured cells were detected, following nuclear staining, using fluorescent microscopy after which the cells were counted using CellProfiler. The total number of captured cells per channel was  $>200$ , which is the minimum number for CD-sens analysis (unpublished data), when the number of cells in the negative control (no capture antibody) were subtracted from the number of cells in the positive control (anti-CD203c antibody). The minimum number of captured basophils is based on empirical data from flow cytometry-based CD-sens experiments.



**Figure 17.** Optimization of basophil capture in a multi-channel microfluidic chip. (a) Image of the multi-channel chip with 16 parallel straight channels with one inlet and eight outlets. (b) Optimization of basophil capture in the microfluidic chip using basophil KU812 cell line ( $n=3$ ) at different capture flow rates (50, 60 or 70  $\mu\text{l}/\text{min}$ ) and with (positive control) and without (negative control) capture antibody (anti-CD203c). (c) The total number of captured cells from blood ( $n=3$ ) was counted after capture with (positive control) and without (negative control) capture antibody. Results are presented as median and interquartile range.

Before the multi-channel can be used to analyze samples from milk allergic patients, we must reduce the background activation seen in paper I and II. Different studies on basophil function and behavior have been carried out in paper III with this purpose. In addition, create an image analysis program that can adjust for the signal intensity could also be a possible solution. The image analysis program CellProfiler has been used to build a pipeline (include relevant modules with specific functions for e.g. cell identification and counting). A pipeline to identify cells stained with nuclear stain and fluorescently labeled anti-CD203c-ab and/or anti-CD63-ab was created to count the number of captured basophils (CD203c+) and activated basophils (CD203c+, CD63+). A threshold was set to distinguish between activated and non-activated CD63-signal since we reported in paper I that the MFI for CD63 is lower in non-stimulated basophils (background activation). Unfortunately, the program was not quite sensitive enough due to variations in signal intensity from fluorescently stained CD203c and CD63. The pipeline needs further optimization, alternatively a different program is needed to enable automated basophil counting. A way around this might be to change detection method. Other microfluidic tests for detection of cell degranulation has been seen to be quite sensitive using e.g. impedance measurements, even though it has not been proven to work with human basophils from blood yet [147]. Impedance measurements was able to detect even slight changes in morphology, which might be due to e.g. piecemeal degranulation. Another possibility would be to detect human basophil degranulation in a chip by using surface plasmon resonance (SPR) [146]. A limitation of SPR is the quite low throughput since the area that can be analyzed is quite small. Alternatively, if we can reduce the background activation a threshold for the image analysis program might not be needed. Another capture antibody can be used to minimize the activation from CD203c crosslinking, e.g. CD193, but since the marker is also expressed on eosinophils it would for example require a negative selection step prior to basophil capture to deplete the eosinophils prior to basophil capture. Since the background activation caused by CD203c crosslinking is quite low, it might be acceptable if it can be reduced in other steps during miBAT processing. Collecting blood in tubes containing EDTA instead of sodium heparin could possibly prevent degranulation prior to allergen incubation [169], as discussed above (paper III).

Further, there are improvements being developed for BAT that does not include microfluidics and might be a good step between standard BAT and miBAT or CD-sens on chip. One example is a 96-well plate that contains dry reagents with a 5-color antibody panel for detection. The test is quite fast and only requires 5 pipetting steps, but the plate needs to be analyzed using flow cytometry containing a plate reader [171], which still does not solve the need for cheap tests and easy access at POC. Alternatively, if the background in miBAT cannot be reduced and an image analysis program cannot be used to solve the background problem, another solution could be to replace the flow cytometer with a microfluidic chip to make the test cheaper and more portable. There would still be manual steps in the laboratory for stimulation and staining, but the analysis of the basophils could be done using a flow cytometry chip. The cell focusing could be done using either sheath fluid like in standard flow cytometry or by using inertial microfluidics or external forces such as dielectrophoretic or acoustics. The detection could be done using microscopy, which will enable detection of fluorescently labeled markers [172]. However, these chips have so far still lower throughput than standard flow cytometry and cannot analyze as many fluorophores simultaneously. There is still optimization needed before flow cytometry on chip is a viable alternative.

In summary, the parallel multi-channel chip will be evaluated using blood from milk allergic children and compared to flow cytometry-based CD-sens. The main challenge with the microfluidic chip is the background activation discussed in paper I and II, which is further addressed in paper III. There is a need for an automated image analysis program that can distinguish between real and background activation if the background cannot be reduced by other means.

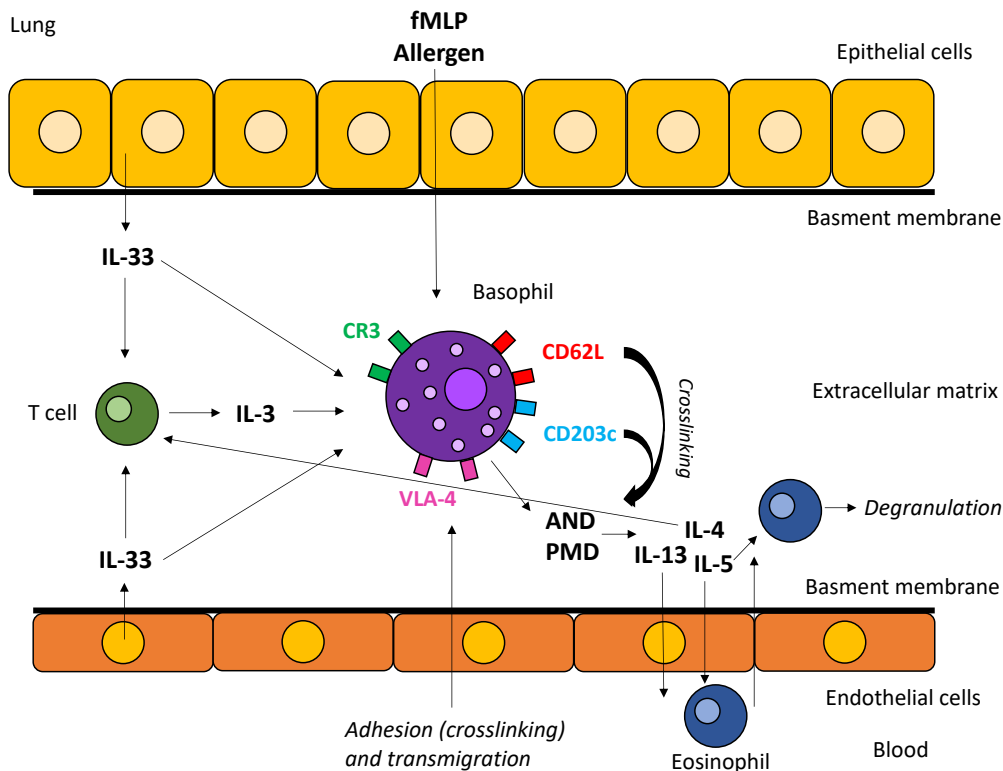


## 5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Allergic diseases affect approximately 30% of the population, impacting their quality of life. Reliable, accessible and easy to use diagnostic methods are therefore warranted. There are both in vivo and in vitro diagnostic tests available, where the gold standard today is in vivo challenge tests. In vitro tests include allergen-specific IgE-ab assays and the basophil activation test (BAT). In BAT, flow cytometry is used to analyze the CD63-expression on basophils from blood that are stimulated with decreasing allergen concentrations (dose-response curve) from which the sensitivity can be calculated. To further increase the accessibility and improve BAT, we developed a microfluidic chip to perform BAT (miBAT) in paper I and II.

We have developed miBAT that is capable of capturing basophils directly from whole blood. The captured basophils from both healthy donors and allergic patients were successfully activated using anti-Fc $\epsilon$ RI as a positive control. Allergic patients showed a dose-dependent activation using allergens, whereas healthy controls showed no significant difference in activation between the negative control and the allergens. The results were compared to flow cytometry-based BAT, where no significant difference in activation between the two tests were detected. It was also possible to distinguish between activation with relevant and non-relevant allergens in allergic patients but due to the difference in milieu during basophil activation between miBAT and BAT, the level of activation (dose-response) was not quite comparable. Together, miBAT could be verified as a test for allergy diagnostics comparable to traditional flow cytometric BAT. The possibility that miBAT can be used at POC, will further expand the availability of the diagnostic test in the future.

We studied the effect that crosslinking of CD203c (mimicking basophil capture in miBAT) had on basophil anaphylactic degranulation (CD63-expression) and thus also on the background activation seen in unstimulated basophils captured in miBAT. CD203c crosslinking was determined as a potential factor for the background activation. We also studied the effect adhesion molecule crosslinking and cytokine stimulation had on basophil degranulation using either anti-IgE-ab or fMLP. We saw that the %CD63-expression was reduced after CD62L and CD49d crosslinking followed by anti-IgE degranulation compared to only anti-IgE degranulation, suggesting a regulatory function of basophil adhesion. This is of interest both in vivo since there is little known about human basophil regulation but also in miBAT since we have seen a lower IgE-dependent activation in miBAT compared to flow cytometry-based BAT. IL-3 and IL-33 priming increased the %CD63-expression caused by fMLP activation, which was to be expected for IL-3 but has to our knowledge not previously been reported for IL-33. Together, this indicates that basophil regulatory functions are involved in degranulation both in vivo as well as in vitro in miBAT. More studies of basophil regulation of IgE-dependent and IgE-independent activation are needed to better understand how adhesion in combination with relevant cytokines affects human basophil degranulation. For example, to better understand the roles of basophils in inflammation it would be interesting to further study sequential stimulation with cytokines, followed by crosslinking of adhesion molecules and IgE-dependent or IgE-independent activation to better mimic basophil recruitment and activation in vivo during inflammation in tissue (Figure 18).



**Figure 18.** Illustration of possible stimuli (crosslinking of adhesion molecules and cytokines) that can affect basophil anaphylactic (AND) and piecemeal (PMD) degranulation from IgE-dependent (allergen or anti-IgE) and IgE-independent (fMLP) activation in tissue.

To enable CD-sens on chip, we have designed and optimized the capture of basophils from both human basophil cell line and whole blood in a multi-channel chip. The multi-channel chip had 16 parallel straight channels with one inlet and eight outlets allowing up to eight different allergen concentrations to be run simultaneously. The next step is to continue to work on both reducing the background activation seen in miBAT, as well as developing and optimizing an image analysis program. A pipeline in CellProfiler is under development to create an automated CD-sens on chip test. We also envision the integration of a gradient generator module to deliver desired concentrations to the captured cells to make the device easy to use for non-skilled personnel. CD-sens on chip will be evaluated using clinical samples from milk allergic children and compared to standard flow cytometry-based CD-sens. The aim is to eventually replace in vivo challenge tests and CD-sens with CD-sens on chip.

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